

THE PHYSIOLOGICAL RESPONSES AND MECHANISMS
OF YOLK PRECURSORS AND EGG PRODUCTION
IN LAYING HENS EXPOSED TO HIGH
AMBIENT TEMPERATURE

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ABSTRACT

Most modern highly productive poultry strains have been developed in temperate countries, with little opportunity for heat tolerance to be a selection factor. When these birds are moved to tropical or subtropical countries their egg production, egg quality, food intake and growth rate decrease at high ambient temperature. Varying reports on the effects of heat stress on egg and yolk production may be attributable to the differences of the range and duration of thermal loads employed, different bird strains, ages, diets and food intake responses. There are, however, only a few studies which attempt to explain the actual physiological mechanisms involved in the adaptation of laying hens to thermal loads, especially those relating to the changes in egg yolk precursors including vitellogenin and very low density lipoprotein (VLDL) synthesis, secretion and deposition in the oocytes.

The major yolk precursors are vitellogenin, a metalbinding protein, and VLDL, a specialised type of triglyceride-rich lipoprotein found in abundance in the plasma of laying hens. Both vitellogenin and VLDL are synthesised in the liver in response to oestrogen stimulation, transported to the ovary and transferred and deposited into the growing oocytes by a selective mechanism. Chronic heat stress in laying hens reduces yolk size and total yolk production considerably. Any changes in yolk precursor synthesis mediating such a response may involve altered oestradiol secretion or reduced sensitivity of the hepatocytes to oestrogen stimulation. Other possible mechanisms of reduced yolk accumulation include disturbances in the transport of vitellogenin in the blood stream and changes in the level of ovarian uptake of egg yolk precursors by the growing oocytes.

The present project examined the changes in egg yolk precursor levels in the plasma of laying hens chronically exposed to different degrees of thermal stress (32 and 35°C with various relative humidities) and precursor uptake by the oocytes. The study also addressed the role of oestrogen in yolk precursor responses to chronic heat stress. In addition, the role of nutritional vitamin E supplementation as a strategy for alleviating the effects of heat stress has been examined. The studies have confirmed that heat stress reduces yolk and egg production concomitant with reduced circulating vitellogenin and VLDL in the plasma. The decreased plasma concentrations of vitellogenin and VLDL resulting from exposure to high ambient temperatures are not simply a consequence of reduced food intake. Studies applying some degree of restriction on feeding the birds (85, 70 and 55%) compared to the control group (*ad libitum*; 100%) and a paired-feeding study, where a control group was fed with the same amount as the heat stressed group consumed were also undertaken.

It was also demonstrated by oestrogen administration *in vivo* under different heat loads (30°C and 80% relative humidity [RH]; 35°C and 55% RH), that oestrogen increases the availability of both vitellogenin and VLDL in the circulation of heat stressed and control (thermoneutral) hens although their responses were not identical quantitatively. There was a doubling of the concentration of VLDL in response to oestrogen treatment during the second week of heat stress. The results of this exogenous oestrogen treatment suggest that the liver is capable of synthesising vitellogenin and VLDL and this also means that hepatocyte sensitivity is not altered during heat stress.

Other experiments in this study were also carried out to determine whether the mechanism mediating reduced egg production in heat-stressed hens involved a change in the rate of uptake of yolk precursors by the oocytes. This led to the establishment and validation of a new technique which allows characterisation of oocyte yolk precursor uptake *in vivo*. The labelling of natural vitellogenin present in high concentrations in fresh laying hen plasma by incubation with ⁶⁵Zn proved extremely effective. The findings indicate that the novel technique is appropriate for the measurement of oocyte vitellogenin uptake *in vivo* and therefore for the elucidation of the mechanisms controlling oocyte development and yolk accumulation in response to environmental challenges.

Further studies have been carried out in an attempt to alleviate the effects of heat stress in laying hens using supplementation of the diet with α -tocopherol (vitamin E), a biological antioxidant. Supplementation of the diet with 500mg/kg vitamin E increased circulating egg yolk precursor concentrations during exposure to high heat loads and this may support the observed improvement in yolk and egg production. It is suggested that vitamin E supplementation represents a possible strategy for reducing the effects of heat stress. The study provides important evidence of a possible physiological basis for the changes in yolk and egg production induced by chronic heat stress in laying hens.

DECLARATION

This thesis has been composed by my self and the work presented herein is entirely my own. This thesis has not been submitted for any other degree elsewhere. All references and assistance from other individuals during the planning, execution and presentation of this thesis are duly acknowledged.

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PUBLICATIONS ARISEN IN COMPLETING THE PHD WORK

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understanding, and encouragement**

ABBREVIATIONS

ALP	alkaline phosphatase	mg	milligram
ANOVA	analysis of variance	min	minute
BSA	bovine serum albumin	MJ	megajoule
Bt	body temperature	ml	millilitre
BW	body weight	mm	millimetre
CA	carbonic anhydrase	mM	millimolar
Ca	calcium	MW	molecular weight
CaCO ₃	calcium carbonate	P	phosphorus
CO ₂	carbon dioxide	<i>p</i>	probability
cpm	counts per minute	P _{CO₂}	(partial) pressure of carbon dioxide
CP	crude protein	Pi	inorganic phosphate
d	day	r ²	correlation coefficient
Da	Dalton	RH	relative humidity
DexSO ₄	dextran sulphate	RIA	radioimmunoassay
df	degree of freedom	rpm	rotation per minute
E ₂	oestrogen	sec	second
EDTA	ethylenediamine tetraacetic acid	SD	standard of deviation
EHP	evaporative heat production	s.e.m.	standard error of the mean
EHL	evaporative heat loss	SHL	sensible heat loss
FSH	follicle stimulating hormone	T3	triiodothyronine
<i>g</i>	gravity	T4	thyroxine
GH	growth hormone	T _a	ambient temperature
h	hour	T _b	body temperature
HCG	human chorionic gonadotrophin	TG	tryglyceride
HS	heat stress	T _r	rectal temperature
iu/L	international unit per litre	TRIS	tris[hydroxymethyl] amino-methane
k	kilo	TSH	thyrothopine stimulating hormone
kBq	kilobeqqurell	v/v	volume per volume
kDa	kiloDalton	Vg	vitellogenin
kg	kilogram	VLDL	very low density lipoprotein
LH	luteinising hormone	w/v	weight per volume
LDL	low density lipoprotein	ZLTE	zone of least thermoregulatory effort
m	meter	ZMM	zone of minimum metabolism
μ	micro	Zn	zinc
ME	metabolisable energy	⁶⁵ Zn	radioactive zinc

CHAPTER ONE

LITERATURE REVIEW: AVIAN FOLLICULAR GROWTH AND PHYSIOLOGICAL RESPONSES TO HEAT STRESS

1.0. INTRODUCTION

Prior to modern commercial genetic selection programmes hens exhibited lower rates of egg production, late onset of sexual maturity, slower growth rate, lower feed conversion efficiencies and longer moulting periods. Modern commercial laying hens may produce 300 eggs per year compared to the 20 - 60 eggs in clutches produced by wild and unselected birds. As a result of selection in standardised high quality environments, modern hens are more susceptible to the changes of environmental conditions and less resistant to diseases than their unselected relatives.

Egg production in laying hens is a complex physiological processes central to which is the regulation of oocyte development and yolk production. The egg yolk precursors, such as lipophospho-proteins, are synthesised in the liver, under oestrogenic control and transported in the circulation to the ovary where they are incorporated into the yolk of growing oocytes (McIndoe, 1971). The major components of yolk precursors in the plasma are vitellogenin and a specialised type of triglyceride-rich lipoprotein. Although the synthesis of yolk is regulated specifically by oestrogen the overall process of vitellogenesis depends upon the gonadotrophins. A major function of oestrogen is to sensitise the hypothalamic-pituitary axis to the positive feedback effects of progesterone in the stimulation of vitellogenesis (Wilson and Sharp, 1976). In addition, follicle stimulating hormone (FSH) and luteinising hormone (LH) not only stimulate endogenous follicular oestrogen synthesis and secretion, but also regulate the uptake of yolk proteins (Follet and Redshaw, 1974).

Decreased egg production could be said in a very simple way to be releasing oocytes at less than the normal rate, regardless of whether it is linked with a decrease in the size or weight of egg, in other words producing oocytes less frequently. This phenomenon could be due to hormonal changes and/or disturbances in the hen's cyclic process of egg production. There are a lot of demands in producing an egg, such as the availability of protein and fatty acids for the yolk and albumen synthesis; enough calcium, phosphorus and carbonate for the production of egg shell in the shell gland and also important is the availability of energy from food intake.

Environmental factors influencing egg production have received considerable attention over the past few decades. Of particular interest have been the effects of heat stress and nutritional modification. Most modern highly productive poultry strains have been developed in temperate countries, with little necessity for heat tolerance to be a selection factor. Therefore when these birds are moved to tropical or subtropical countries appropriate management and physiological acclimatisation must provide them with protection against the heat, although their productivity may be impaired in the process.

Yolk production is influenced by a variety of biological and environmental factors, such as genetic strain of the birds, geographical influences, and composition of the feed (Nemecz and Mennear, 1995). It is well known that heat stress reduces egg production, including egg size and shell thickness, while improving food efficiency (De-Andrade *et al.*, 1977; Miller and Sunde, 1975; Deaton *et al.*, 1981, 1982; Yamada and Tanaka, 1988). Egg production is more affected by constant high ambient temperatures than by cyclic high temperatures (Payne, 1966; Miller and Sunde, 1975a; Yamada and Tanaka, 1988). It has been suggested that the reduction in egg production is not only due to a significant reduction in food intake, but is also a direct effect of the heat exposure on the hens (Francis *et al.*, 1991). Although Payne (1966) and De-Andrade *et al.* (1976) have suggested that the detrimental effects of heat stress on egg production could be minimised by increasing energy levels in the diet in order to benefit from the inadequacy of regulation of energy consumption as dietary energy level is changed, the internal mechanisms involved have not been explained clearly. Yet it is recognised by Miller and Sunde, (1975) and Redshaw and

Follett (1972) that there are some changes in the neuroendocrine system in response to high environmental temperatures, especially those affecting the hypothalamus and the pituitary glands. These changes may result in altered production of gonadotrophins and steroid hormones. This is particularly important in relation to egg production in laying hen considering that the endocrine system regulates the synthesis of egg yolk precursors and probably its deposition in the ovarian follicle.

Other aspects of egg production may be affected by chronic heat stress. Changes in acid-base balance due to panting in heat stressed birds result in elevation of blood pH which in turn reduces the blood ionised calcium pool; ionised calcium is the biological form used by the avian shell gland to produce egg shell. Organic fatty acids, lactate and pyruvate, produced during thermal panting increase the complexed calcium, therefore further restricting the available calcium for egg shell formation. As reported by Odom *et al.* (1986), a persistent blood alkalosis was developed in heat stressed Leghorn hens when temperature reached 35°C. Accompanying this steady rise in arterial pH there was a decrease in the ionised calcium concentration. The arterial P_{CO_2} exhibited a decline to a low value two hours after the start of the heat exposure. At this point in time, the ionised calcium level was reduced by 15% and there was an increase in pyruvate and lactate.

It is apparent that a number of physiological mechanisms may be affected by chronic exposure to heat stress and these will exert complex influences on several aspects of oocyte development, egg formation and production. The synthesis and availability of egg yolk precursors for transport to and incorporation into the developing oocyte have not been previously examined in heat stress laying hens. The present studies have addressed this topic.

It can be suggested that reduced hepatic synthesis of egg yolk precursors may restrict uptake by the oocyte thus decreasing the rate of oocyte growth. This phenomenon may be reflected in changes in the circulating levels of the precursors. The uptake process of these materials at the oocyte membrane may also be altered during heat stress. Any changes in rate or affinity in the transport systems may represent adaptations which may either sustain oocyte development or further

decrease the rate of precursor accumulation. The relative contributions of such functional changes have not been characterised in previous studies.

Through a knowledge of the physiological mechanisms mediating changes in oocyte development and egg production it may be possible to design nutritional and husbandry strategies to minimise the detrimental effects of elevated environmental temperatures and maximise productivity. In the present studies the possible benefits of antioxidant supplementation have also been considered in the relation to egg production during chronic heat stress. A consequence of prolonged hyperthermia may be excessive membrane lipid peroxidation, membrane damage and cellular dysfunction. Such mechanisms could mediate changes in hepatic yolk precursor synthesis and/or oocyte membrane transport and yolk accumulation. The effects of addition of α -tocopherol (vitamin E), a recognised biological antioxidant, to the diet, were therefore investigated. Vitamin E may offer “membrane protection” by both direct antioxidant effects and facilitative interactions with selenium containing glutathione peroxidase.

The work described herein should provide an improved understanding of the physiological mechanisms mediating decreased egg production in the heat stressed laying hen and provide a sound scientific basis for strategies for the amelioration of these detrimental effects.

1.1. The objectives of the thesis

Although the effects of heat stress have been reported as reducing egg numbers, egg size and rhythm of egg production, the precise mechanisms involved remain unknown. Questions which remain are do the hens still have their follicle hierarchy available as in normal birds or do the follicles grow more slowly, and/or is the pattern of regulation of development and yolk production altered during heat stress? It is well known that heat stress reduces food intake, but whether this reduction explains all of the decrease in egg production is uncertain. Egg production may be determined by the rate of oocyte development and of yolk production; and

since the vast majority of oocyte contents are egg yolk precursors which are taken up from the blood circulation, the availability of egg yolk precursors is vital. This leads to an attempt to answer the questions; what system might alter during exposure to heat stress, whether the oocytes grow slowly, whether there are disturbances in egg yolk precursor transport in the circulation or in the synthesis levels, and the possibility of changes in rate of uptake by the oocytes.

Considering the scientific and commercial importance of expanding the present knowledge on the effect of heat stress in laying hens and the physiological responses of the bird to such adverse conditions, this project was aimed at characterising the responses and mechanisms of changes in oocyte development and yolk production in laying hens chronically exposed to high thermal loads. These included the evaluation of the egg yolk precursors in the circulation, the uptake by the oocytes, the role of oestrogen, and determination of some of the physiological responses which alter egg development and production. This study concentrated upon changes in the plasma concentrations of vitellogenin and VLDL as the major egg yolk precursors, both total and ionised calcium, alkaline phosphatase activity and inorganic phosphorous in laying hens during exposure to chronic heat stress. The effects of nutritional vitamin E supplementation in the diet, as one of possible solutions for alleviation of the effects of heat stress and for the improvement laying performance, have also been investigated.

1.2. THE PHYSIOLOGY OF THE OVARY AND YOLK SYNTHESIS IN DOMESTIC FOWL

The domestic hen has a single functional ovary which is located in the anterior of the body cavity, ventral to the aorta, caudal to the vena cava and adjacent to the cranial extremity of the left kidney and the caudal part of the lung. The distribution of germ cells between the right and left ovary in early embryogenesis is already unequal, with more colonising the left ovaries, which then becomes larger, than the right one (Gilbert, 1979), which does not develop fully. Until 4 months the left ovary grows

slowly but as the hen becomes sexually active the ovary grows enormously from around 0.5 to 60 g.

When oogonia, the rapid multiplying primordial germ cells, reach the prophase of their first meiotic division they become primary oocytes (Franchi *et al.*, 1962), this occurs around the time of hatching. The granulosa cells, derived from the embryonic ovarian epithelium, align themselves around oocytes between the 4th and 6th day after hatching (Romanoff, 1960; Franchi *et al.*, 1962).

1.2.1. Follicular structure and hierarchy

The hen's ovary is extremely well vascularised, having extensive arterial and venous systems. A striking feature of the ovary is the complex and very extensive neural tissue (Gilbert, 1979). The adult ovary may contain several million oocytes, all of which are formed during embryogenesis (Franchi, *et al.*, 1962). Hundreds of these can be seen with the naked eye, though there are usually less than 100 with diameters greater than 1 mm. Most of the latter are usually described as "white" follicles since the yolk material is very pale in colour. A characteristic feature of the ovary of a laying hen is the hierarchical structure of the follicular population. The hierarchy usually consists of 4 to 6 developing follicles in increasing (Figures 1.1 and 1.2), but the number varies and is related to the pattern of laying; each member is usually formed at intervals of about 24 h.

The growing follicle consists of a centrally placed oocyte, containing yolk material and the living cytoplasm. The nucleus of the oocyte appears to remain in the diplotene stage until just before ovulation, when the first maturation division occurs. The second maturation division occurs after ovulation. In the early stages, from about 1 to 3.5 mm diameter, the primordial (white) yolk is formed (MacKenzie and Martin, 1967; Griffin *et al.*, 1984). Thereafter both primordial and "yellow" yolk are accumulated for a time, though the production of primordial yolk stops when the follicle reaches about 7 mm diameter.

1.2.2. Follicular growth and development

Definitive yolk spheres first appear in oocytes of about 2.5 mm diameter, and thereafter form the bulk of the cell, which in turn forms the bulk of the ovarian follicle. In the first vitellogenic phase (2.5 to 4 mm) primordial yolk is laid down. It is followed by a transitory phase (4 to 8 mm) marking the onset of formation of the predominant type of yolk. The process gathers momentum in the main vitellogenic phases and results in growth of the follicle from 8 to 37 mm in diameter over a comparatively short period. The follicles in the three phases are white, yellowish and deeper yellow, respectively. Hence the yolk deposited in the last two phases is generally referred to as "yellow" yolk (Griffin *et al.*, 1984).



Figure 1.1. The normal hierarchy in a laying hen with six developing follicles.

Some studies on the growth of the follicular hierarchy indicate that a follicle takes between 5 and 6 d to increase in size from about 1 to 8 mm and 3 to 4 d to increase in size from 3 or 4 to 8 mm. Since probably 1 d is required to grow from 6 to 8 mm; an average increase in the diameter of 1 mm/d seems a reasonable assumption on present information for the smaller follicles. The small "white" follicles (about 2-5 mm in diameter) form a "pool" from which one is selected at random to enter its "rapid-growth phase", although the validity of such a concept has been questioned (Gilbert, 1971; see **Figure 1.2.**). The "pool" view is probably untenable since the small follicles are also growing rapidly. The number of follicles in the currently accepted hierarchy, and which are related to the number of eggs produced (Gilbert *et al.* 1980), will depend on the difference between the rate of production of the small follicles of 1 mm (or less) and the rate of atresia thereafter.

1.2.3. Yolk precursors synthesis and deposition

At peak reproductive performance the ovary usually contains five to six yellow follicles in a graded size sequence. Their growth characteristics have been determined from the ring pattern formed in the yolk after the administration of lipid-soluble dyes to the birds at regular intervals. Most of the growth occurs in the 7 to 11 d before ovulation (Gilbert, 1971), in radial increments of about 2 mm per day in follicles of 7 g, and continues in most oocytes until 2 to 3 h before ovulation (Gilbert, 1971).

The presence of the gonadotrophic hormones, FSH and LH in the circulatory system results in the synthesis and secretion of oestrogens, particularly oestrone and oestradiol 17- β from the granulosa cells of the ovarian follicle (Redshaw and Follett, 1976). Oestrogens act on the liver to stimulate the synthesis and release of vitellogenin which is the major protein constituent of the yolk granule. Oestrogen increases the synthesis of a β -lipoprotein, this protein is central in the transport of the lipids to the oocyte (Redshaw and Follett, 1976; Wahli *et al.*, 1981).

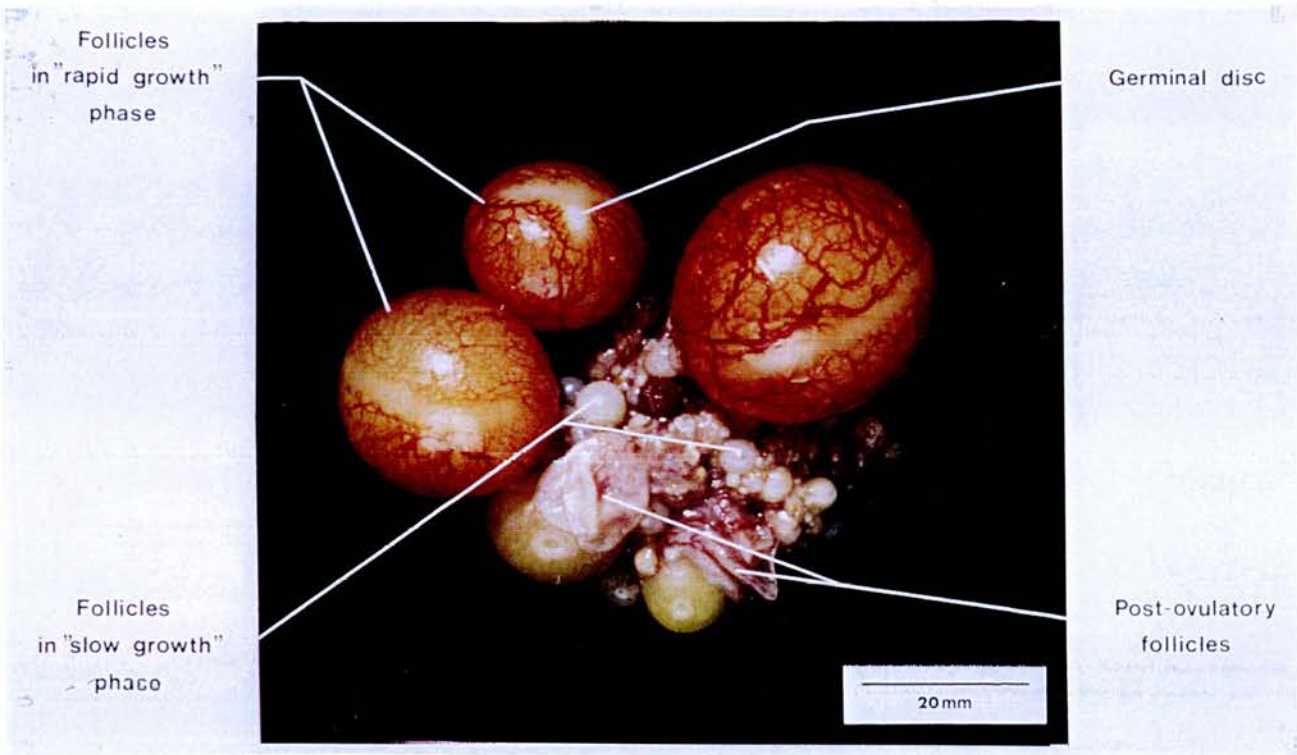


Figure 1.2. An ovary of a laying hen with a hierarchy of growing oocytes. Notice the small white follicles in the centre as the pool from which one follicle is selected to enter the rapid-growth rate.

In the early phases of vitellogenesis, while the yolk laid down in the primordial and transitory phases amounts to less than 2% of the total yolk mass, events associated with its formation may have a bearing on the mechanisms controlling the initiation of vitellogenesis. However, little is known about the chemical nature of primordial yolk apart from the fact that it is essentially proteinaceous (Bellairs, 1965). In contrast to the main vitellogenic phase, structures originating in the ovary play a greater part in the formation of this yolk (Griffin *et al.*, 1984). During the vacuolar stage, complex macrobodies are formed in the superficial cytoplasm from the endocytosed material. Entering the transitory phase, there is a gradual cessation of the phagocytosis of lining bodies and an increase in the rate of uptake of some yolk precursors by pinocytosis. The majority of developing follicles become atretic during the course of this period and are rapidly resorbed. Thus, atresia appears to be the normal fate of most follicles in the early vitellogenic phases.

1.2.3.1. Biochemistry of yolk proteins

Proteins from the yolk can be isolated which usually involves the separation of three yolk fractions by centrifugation after dilution with water or saline. These three fractions are phosphoproteins (phosvitin, lipovitellin and vitellogenin), triglyceride-rich proteins (yolk and plasma triglyceride-rich proteins) and yolk water-soluble proteins (α -, β - and γ -livetins).

The major protein of hen's egg yolk is phosvitin which is a highly atypical protein (Griffin *et al.*, 1984). More than 50% of its amino acids are serine residues, with 90 to 95% of them phosphorylated (Rosenstein and Taborsky, 1970). Phosvitin also contains at least one phosphothreonine residue (Clark, 1970) and a carbohydrate side-chain. Phosvitin accounts for about 80% of the protein-bound phosphorus of yolk, but only about 10% of the yolk protein. Phosvitin can bind a variety of multivalent metal ions, including Ca^{2+} , Mg^{2+} and Fe^{3+} (Grizzuti and Perlmann, 1973). Most of the Ca^{2+} and Fe^{3+} found in yolk is present in the granule fraction, presumably bound to phosvitin (Clark, 1974), whilst Mg^{2+} does not seem to have been reported.

Lipovitellin. The amino acid compositions of α - and β -lipovitellin are very similar and the major difference between them is in the phosphorus content of their protein moieties: 0.5% for α -lipovitellin and 0.27% for β -lipovitellin. Beta-lipovitellin appears to be preferentially incorporated during yolk deposition in the early vitellogenic phase. Both of them normally exist as dimers which can be dissociated into identical monomers with molecular weights (MW) of about 200 000. These contain 15 to 20% lipid and polypeptide components must therefore have a total MW of about 170 000.

Vitellogenin. The synthesis of vitellogenin is regulated by oestrogen and as a metalbinding protein, vitellogenin is rich in calcium and zinc (Taylor and Dacke, 1984). Plasma concentration of protein-bound phosphorus increases markedly at the onset of lay or after administration of oestrogen to chicks. Early studies indicated that the plasma phosphoprotein was not identical to egg yolk phosvitin (Beuving and

Gruber, 1971). Subsequent studies have shown that the phosvitin and lipovitellin of both avian and amphibian yolk are derived from a single plasma precursor, vitellogenin (Bergink and Wallace, 1974; Deeley *et al.*, 1975; Jackson *et al.*, 1977; Gordon *et al.*, 1977). There is a strong evidence that phosvitin and lipovitellin are integral parts of the vitellogenin polypeptide (Deeley *et al.*, 1975). The similarity of the products of hydrolysis of vitellogenin by different types of proteolytic enzymes suggests that only a few of the peptide bonds are susceptible to hydrolysis (Deeley *et al.*, 1977). The model proposed by Gordon *et al.* (1977) predicts the two phosvitins should occur in equimolar proportions in yolk, but Clark (1970) found a ratio of 2:1 in favour of the larger phosvitin.

The molecule of vitellogenin can be proteolytically cleaved to produce lipovitellin and phosvitin in the ratio of 1 to 2 in each monomer (Griffin *et al.*, 1984; Griffin, 1985). Lipovitellin may also be divided into distinct a- and b-lipovitellin units (Burley and Cook, 1961) and phosvitin consists of a number of phosphoglycoprotein of different molecular weights ranging from 13 to 40 kDa (Wallace and Morgan, 1986).

Very low density lipoprotein (VLDL). Another egg yolk precursor also found in abundance in the plasma is yolk triglyceride-rich lipoproteins. The laying hen's plasma contains a high concentration of triglyceride-rich lipoproteins. These are similar to the yolk triglyceride-rich lipoproteins and there is a direct transfer of lipoproteins from plasma to yolk (Griffin *et al.*, 1984). The size distribution of plasma lipoproteins is similar to that of yolk LDL2 lipoproteins (Bacon *et al.*, 1973). The lipid and fatty acid compositions of plasma triglyceride-rich lipoproteins are virtually identical to those of yolk triglyceride-rich lipoproteins (Christie and Moore, 1972), but Gornall and Kucsis (1973) found differences in the relative proportions of their dienoic and trienoic glycerolipids. Plasma and yolk VLD lipoproteins contain common antigens (Hillyard *et al.*, 1972; Yu and Marguardt, 1973). The triglyceride-rich lipoproteins of the low density fraction of yolk contain about 65% of the yolk solids and 95% of the yolk lipid. Their lipid composition, lipid/protein ratio and lipoprotein density vary with lipoprotein size and diet. Two populations of yolk

triglyceride-rich lipoproteins, low density lipoproteins LDL1 and LDL2, which differ mainly in size, can be separated by centrifugation (Martin *et al.*, 1959; Bacon, 1973) and also by gel filtration (Griffin *et al.*, 1984). They have estimated mean MW of 10×10^6 and 3 to 5×10^6 respectively.

Water-soluble proteins. The major water-soluble proteins of hen's egg yolk were originally designated α -, β - and γ -livetins by Martin *et al.*, (1957). The major constituent of α -livetins is plasma albumin (William, 1962), while β -livetins is heterogeneous, although its components may be immunologically related (McIndoe, 1971). Relative to albumin, there is a greater abundance of α -glycoprotein in yolk than plasma, which suggests it may be actively incorporated into the oocyte. Two additional yolk water-soluble proteins designated as the higher MW protein d-livetins and apovitellenins II, a protein previously found associated with yolk triglyceride-rich lipoproteins. The soluble fraction of yolk also contains a number of quantitatively minor proteins which seem to be responsible for transfer of vitamins into the egg. They also contain transferrin, a glycoprotein with a MW of 77000. Each molecule binds two iron atoms, but it does not represent a significant amount of iron transfer to the yolk. It is probably incorporated bound to vitellogenin.

1.2.3.2. Hepatic synthesis of yolk precursors

Except immunoglobulins, all the major plasma proteins incorporated into yolk are synthesised in the liver. The major yolk precursors, vitellogenin and VLDL, and three of the minor components of yolk, the binding proteins for biotin, thiamin and riboflavin, are either absent from the plasma of immature hens, or present in only very low concentrations (Deeley *et al.*, 1977; Wiskocil *et al.*, 1980). It would therefore, be incorrect to view them only as egg-specific proteins since they may have functions unrelated to yolk deposition (Murthy and Adiga, 1978). The hepatic synthesis of vitellogenin and apo-VLDL-II appears to be completely dependent on oestrogen, since neither protein is normally synthesised in rooster liver (Deeley *et al.*, 1977;

Wiskocil *et al.*, 1980). The plasma concentrations of each of these increases dramatically after treatment with oestrogen (Wiskocil *et al.*, 1980).

The accumulation of triglyceride in the plasma of the laying hen is a result of an oestrogen-induced increase in the rate synthesis of VLDL in the liver (Kudzma *et al.*, 1975) and decreased rate of clearance of VLDL triglyceride from the circulation. VLDLs from the plasma of laying hens or oestrogen-treated chicks are different to those from immature hens or roosters: they are smaller, have a lower electrophoretic mobility (Evans, 1974) and a substantially different lipid composition (Gornall and Kucsis, 1973). However, it is probable that the differences in lipid composition between VLDL from immature and laying hens are primarily due to oestrogen-induced changes in the pattern of lipid synthesis (Griffin *et al.*, 1984).

1.2.3.3. Uptake of yolk precursors

Most of the components of the "yellow" yolk are synthesised in the liver and are carried via the vascular system to the ovary. They then escape through fenestrations in the terminal capillaries, pass between the cells of the theca interna and traverse the basal lamina, which acts to restrict the passage of particles greater than 40 nm. The yolk material then flows between the granulosa cells, through the interstices of the perivitelline layer and comes into contact with the surface of the oocyte (Perry and Gilbert, 1980). Incorporation of the major yolk precursor VLDL is accomplished by receptor-mediated endocytosis (Griffin *et al.*, 1984).

Plasma macromolecules have to cross three potential barriers of the follicle wall before reaching the oocyte: the capillary wall, the granulosa basal lamina and the granulosa layer (Griffin *et al.*, 1984). The vasculature is designed to slow down the blood flow in the follicle and so allow the optimum efflux of plasma to the surrounding tissue spaces, although there is no direct physiological evidence for this and evidence suggests the flow is high (Scanes *et al.*, 1982). A leaky capillary system in the middle and inner thecal zones of yellow follicles has been reported (Perry *et al.*,

1978) which indicates that the capillaries are permeable to plasma components of considerable size and that plasma passes directly into the pericapillary spaces. Such holes in the capillary wall would facilitate the massive transfer of lipoprotein and other yolk components from the circulation to the interstitial fluid. Since fenestrations occur in the terminal capillaries, it must be accepted that blood can pass freely from them, and plasma components and erythrocytes are a common feature of electron microscopy of this region (Perry and Gilbert, 1980). However, only certain plasma components finally enter the oocyte and the concentrations of some of these are much lower in yolk than in plasma while others are selectively concentrated (Griffin *et al.*, 1984).

1.2.3.4. Mechanism of yolk deposition

Proteins enter many cells by the mechanism of receptor-mediated endocytosis. This process involves firstly the specific adsorption of a molecule to its recognition site on the plasma membrane and secondly the internalisation of the complex by means of coated pits. The pits pinch off the plasma membrane and carry the adsorbed protein to locations which differ according to the function of the particular cell. There is no direct evidence for specific receptors for other yolk precursors, though the concentration of the vitamin-binding proteins into yolk strongly suggests that they are incorporated by receptor-mediated endocytosis (Griffin *et al.*, 1984; see **Figure 1.3.** for details). The uptake of some yolk proteins that are not glycoprotein could be dependent on their association with those that are glycoprotein. It is proposed that incorporation of thiamin-binding protein was dependent on its association with riboflavin-binding protein but Miller *et al.*, (1981) showed this was not the case by demonstrating that the riboflavin-deficient eggs laid by hens had normal levels of thiamin.

Endocytosis in oocytes in the main vitellogenic phase takes place solely by means of coated pits (Perry *et al.*, 1978; Griffin *et al.*, 1984). In this phase, the fully formed vesicles are usually located close to the plasma membrane and connected to it

by narrow necks; once separation is complete, it would appear that the vesicles undergo a rapid change in morphology (Perry *et al.*, 1978). The enhancement of endocytotic activity at the onset of the rapid growth phase is indicated by a rise in the uptake of phosvitin and a concomitant increase in the number and size of coated vesicles (Griffin *et al.*, 1984). Though gonadotrophins are known to be essential for the normal growth of the follicle (Lofts and Murton; 1973), the way they operate at the cellular level is still poorly understood.

The yolk spheres of yellow follicles, ranging between 20 to 150 μm , are suspended in a continuous phase fluid and bounded by a trilaminar membrane of dense granular material and embedded in a compact mass of lipoprotein particles (Griffin *et al.*, 1984; see also **Figure 1.3**). Immediately after the coated vesicles are pinched off from the plasma membrane, the lumen is obliterated and the cytoplasmic coating is shed. The membrane-bound spheres formed as a result of these changes are packed with particles. They undergo successive fusion to give rise to yolk spheres of increasing size. When dye tracers are administered orally, to determine the time required for yolk material to be extracted from the plasma, they take about 3 to 5 h to enter the yolk, but give shorter time intervals of 30 to 60 min when administered by injection. The transport of material into the oocyte and its deposition in yolk spheres is an extremely rapid one particularly in the early vitellogenic phases (Griffin *et al.*, 1984).

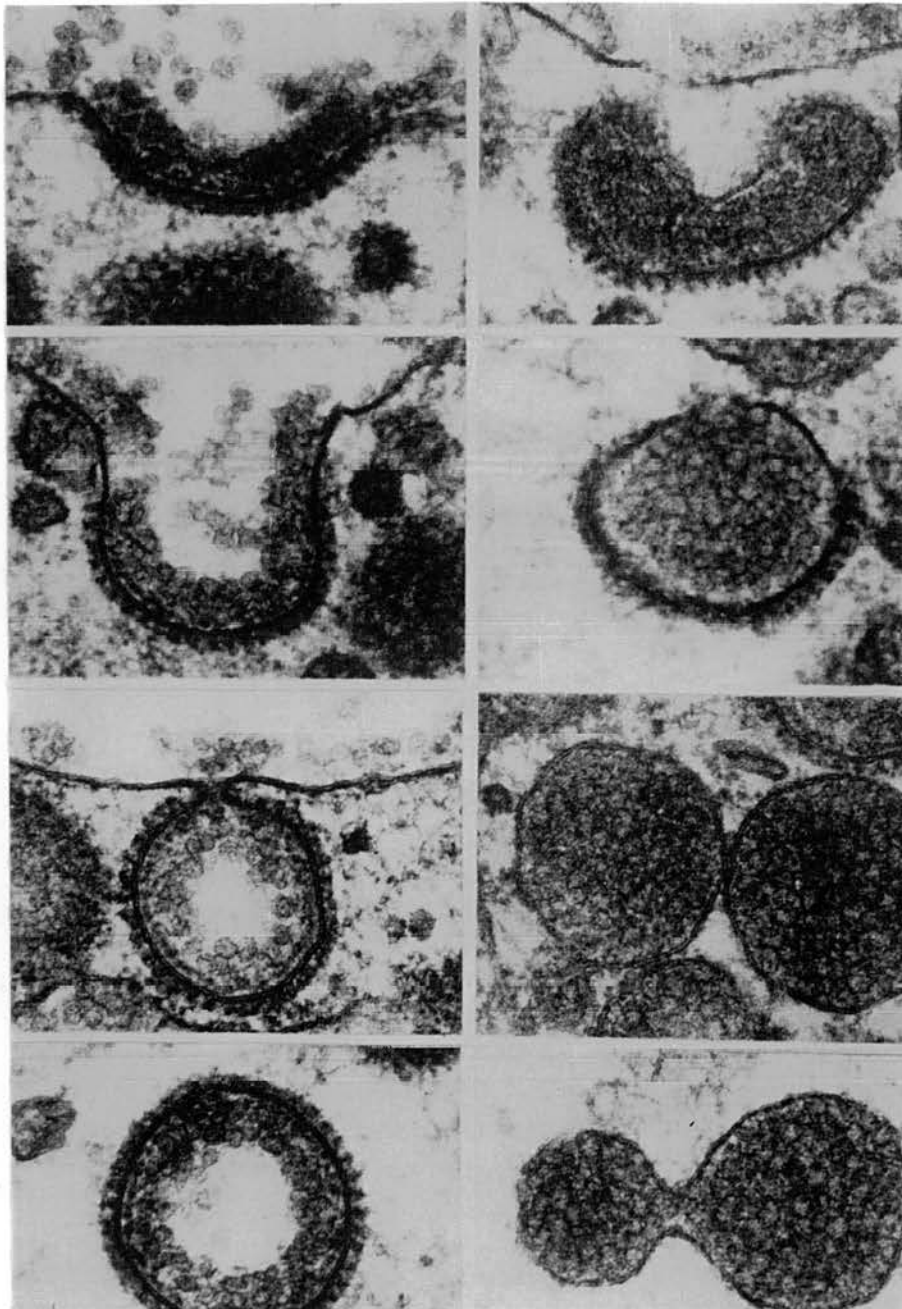


Figure 1.3. Mechanism of receptor-mediated endocytosis of yolk deposition. From the top to down left side show the specific adsorption of the molecules to the site of plasma membrane and their internalisation of the complex by means of coated pits from the cell surface. From the top to down right side show coated vesicles are transformed into solid bodies which then fuse to form small yolk spheres (photo courtesy of Griffin, *et al.*, 1984)

1.2.3.5. Determination of plasma vitellogenin

Vitellogenin synthesis is regulated by oestrogen and the process is stimulated by sexual maturation (Tata and Smith, 1979; Wahli *et al.*, 1981). The plasma concentrations of the lipoprotein increase prior to yolk deposition and the first oviposition and are maintained throughout the laying cycle (Redshaw and Follet, 1976). Plasma vitellogenin is thus regarded as a useful indicator of exogenous vitellogenesis or the reproductive state of the adult females of oviparous species (Copeland and Thomas, 1988). In addition this parameter may be used to characterise reproductive adaptation to change in physiological status or environment change (Phillips *et al.*, 1985).

Some different techniques have been involved in the determination of plasma vitellogenin concentration. These include specific radioimmunoassays (Redshaw and Follet, 1976; Copeland and Thomas, 1988) and indirect methods including measurement of phosphoprotein and calcium as described in fish (Tinsley, 1985). Indirect methods utilise the properties of vitellogenin associated with its function as the precursor of mineral storage proteins in yolk. Following sequestration of vitellogenin by the developing oocyte the molecule is proteolytically cleaved to produce lipovitellin and phosvitin in the ratio of 1 to 2 in each monomer (Cook, 1961; Butler 1983; Griffin *et al.*, 1984; Griffin 1985). Lipovitellin may be divided into distinct a- and b-lipovitellin units (Burley and Cook, 1961) and phosvitin consists of a number of phosphoglycoprotein of different molecular weights ranging from 13 to 40 kDa (Hegenenauer *et al.*, 1977; Wallace and Morgan, 1986). Other authors have used different properties of vitellogenin to determine its circulating levels such as phosphorylated serine residues (Butler, 1983), phosphate content (Deeley *et al.*, 1975), vitellogenin as circulating calcium transporter (Guyer *et al.*, 1980; Parson and Combs, 1981) or as a lipovitellin-phosvitin complex (Robinson *et al.*, 1979).

Specific antibodies to vitellogenin for its determination by radioimmunoassay (RIA) are unavailable. In the absence of enzyme linked immunoprecipitation assays for this protein indirect measurements of its concentration must be employed. In the

laying hen the labile nature of plasma calcium as a result of its metabolism from bone to meet shell synthesis requirements precludes general use of parameter for the estimation of vitellogenin (Taylor and Dacke, 1984).

Another possibility, which has been previously investigated is the use of plasma zinc concentration as an index of vitellogenin production as described by Mitchell and Carlisle (1991). They showed that circulating levels of vitellogenin may be determined by measurement of plasma zinc in the laying hen. The technique is accurate, simple and rapid and does not require the production of purified and radiolabelled vitellogenin or specific antisera as is the case for radioimmunoassay (Redshaw and Follet, 1976) or the electrophoretic separation or lengthy alkali digestions and oxidations of protein required by other indirect methods (Tinsley, 1985). Further, Mitchell and Carlisle (1991) also demonstrated that zinc concentration exhibits a high degree of correlation with plasma organic phosphorous and those of another major egg yolk precursor, VLDL in immature female birds, during sexual development and egg production in mature birds. Plasma zinc is reduced at moult following cessation of egg laying. They concluded that measurement of plasma zinc provides a simple and accurate technique for the estimation of vitellogenin production and reproductive status in the domestic fowl.

1.2.4. Atresia

The majority of developing follicles become atretic and are rapidly resorbed. Thus, atresia appears to be the normal fate of most follicles in the early vitellogenic phases. Atresia in avian follicles is characterised macroscopically by an irregular surface to the follicle. Internally the oocyte is much shrunken, with a ruptured and folded granulosa layer and there is accumulation of yolk material, especially within the connective tissue coat. The first macroscopic signs of atresia are the haemorrhages on either side of the stigma; whether the disruption of the blood vessels is the initial step in atresia has not yet been determined (Gilbert *et al.*, 1985).

The main feature of poor laying birds is a reduction in the ovulation rate due to the loss of large follicles (>8 mm diameter) by atresia, an event seen rarely in birds with good laying performance (Griffin *et al.*, 1984). As atresia is also the normal fate of most of the small follicles, the mechanisms controlling atresia in the small follicles and the large follicles appear to be independent. There may be several possible explanations for this effect; (a) yolk deposition may be slower, leading to a longer time for follicular maturation; (b) there may be a decrease in the number of follicles initially starting growth in a given time; (c) there may be a higher incidence of atresia; and (d) there may be some malfunction of the oviduct resulting in internal laying.

Gilbert *et al.* (1983) suggested that the domestic fowl has two primary control mechanisms regulating ovarian function, namely the regulation of the number of follicles initially starting growth and the regulation of the rate of atresia in the small follicles. Furthermore Waddington *et al.* (1985) showed that under a wide range of conditions the ovary maintains its ability to produce follicles of all sizes. Under adverse conditions it has another way of modifying the ovulation rate by eliminating the large follicles through atresia.

1.2.5. Ovulation

The growing oocyte is surrounded by several layers of supportive tissue totalling about 0.2 mm in depth (Gilbert, 1979). The outer layer is, composed of the theca externa and the theca interna and is well vascularised, with capillaries extending as far as the border with the underlying granulosa layer (see **Figure 1.4** for illustration). Under standard conditions of light and darkness (16L:8D) the occurrence of ovulation in the domestic hen, domestic duck, turkey and Japanese quail is restricted to an 8-10 h period of the day. Ovulation in the hen is preceded 8-4 h earlier by regular increases in the plasma concentration of luteinising hormone (LH), progesterone, testosterone and oestradiol-17 β , although a more gradual increase in the plasma concentrations of testosterone and oestradiol has been observed as early as 18 h before ovulation. There is a substantial evidence that progesterone stimulates

the cyclic release of LH while oestrogen is essential for "priming" the positive feedback mechanism, probably by stimulating the formation of progesterone receptors.

The 8-10 h period of the day to which the occurrence of the preovulatory release of LH is restricted is termed the "open period" and possesses circadian characteristic; it is entrained primarily to the onset of darkness. An increase in corticosterone is associated with the beginning of the "open period" and would appear to be important in the timing of the preovulatory release of LH (Senior and Cunningham, 1974).

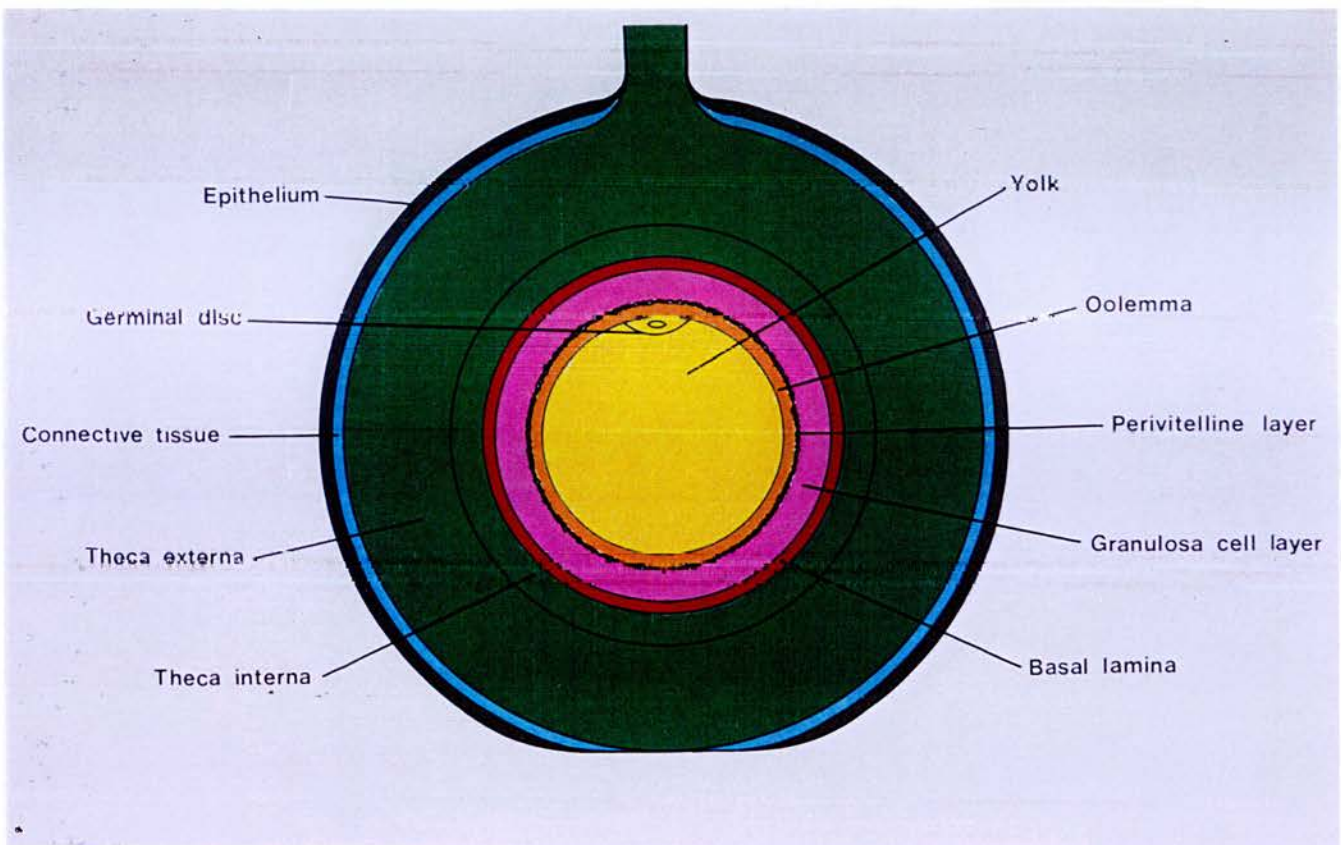


Figure 1.4. A diagram of the mature follicular structure which consists of six distinct and characteristic regions (drawing is not to scale).

1.3. ENDOCRINOLOGY OF THE OVARY

1.3.1. The ovulatory cycle

It has been well documented that the preovulatory surge of LH in the hen is associated with increased plasma concentration of steroid hormones (Furr *et al.*, 1973; Senior and Cunningham, 1974; Etches and Cunningham, 1977) and that exogenous LH stimulates the granulosa cells to enhance the production of progesterone (Hammond *et al.*, 1981). From these facts it has been generally accepted that progesterone exerts a positive feedback effect on the secretion of LH, resulting in the preovulatory surge of the two hormones. Ovulation is induced by progesterone alone possibly in the absence of preovulatory gonadotrophins and progesterone acts directly on the ovary to induce follicle rupture (Nakada *et al.*, 1994).

The events of the ovulatory cycle in poultry occur over hours whereas in mammals they occur over a more prolonged period, because the process of ovulation and the associated ovarian steroidogenesis is different (Gilbert, 1971). In mammals the cycle is composed of follicular and luteal phases, whilst in poultry there is no luteal phase. In poultry it has been determined that pre-ovulatory peaks of oestrogen, progesterone and LH occur simultaneously, approximately 4-6 h prior to ovulation (Wilson and Sharp, 1973; Johnson and Van Tienhoven, 1980).

1.3.2. The role of gonadotrophins in ovarian function

Ovarian hormones profoundly affect the reproductive state of the animal. Although affecting mainly the reproductive organs, they are responsible for the manifestation of secondary sexual characters and often have marked effects on the metabolism and behaviour (also see **Figure 1.5**). Follicle stimulating hormone (FSH) induces the theca to secrete predominantly androstenedione. Oestradiol is also secreted by the theca of domestic hens when stimulated with ovine FSH (oFSH), with

calcium as an important mediator (Ognasbesan and Peddie, 1989). A rise in concentration of circulating FSH at approximately 14-15 h prior to ovulation has been reported (Scanes *et al.*, 1977). However, the measurement of chicken FSH (cFSH) is not very reliable therefore the precise role of this gonadotrophin in pre-ovulatory events is not yet clear. The presence of FSH receptors in the ovary has led to speculation that it is involved in follicular selection and growth (Ritzhaup and Bahr, 1987). FSH and LH significantly promote the proliferation of chicken granulosa cells *in vitro* (Yoshimura and Takamura, 1988). In addition, they also showed that dibutyryl cAMP has a similar effect. Luteinising hormone stimulates production of oestradiol and andostenedione in theca cells (Marrone and Hertelendy, 1985). LH also causes increased progesterone secretion by granulosa cells and progesterone itself is responsible for inducing a pre-ovulatory LH surge and ovulation in the chicken (Johnson and Van Tienhoven, 1984; Robinson and Etches, 1986).

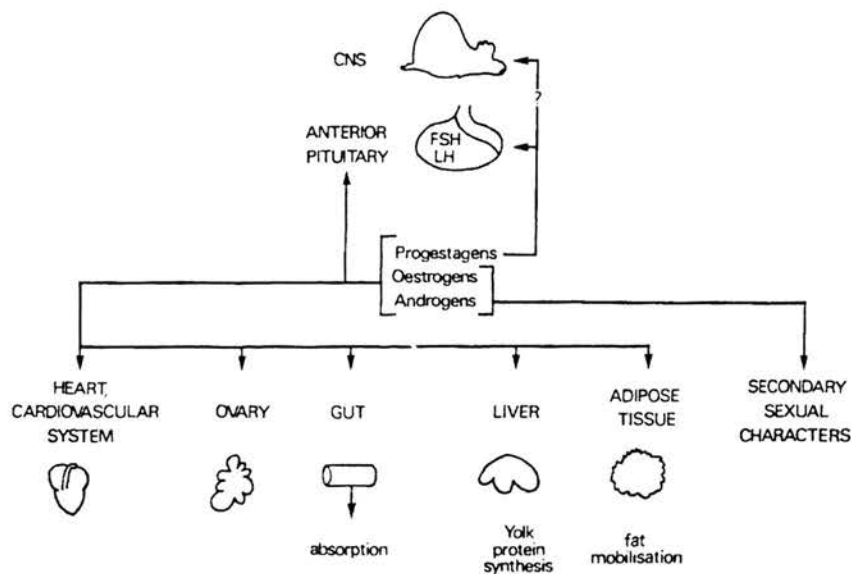


Figure 1.5. Ovarian steroids have widespread effects on the hen's physiology (from Gilbert *et al.*, 1985).

1.3.3. Steroidogenesis in follicular maturation

Oestrogens are mostly produced by the small ovarian follicles whereas progesterone is produced by granulosa cells of the five largest pre-ovulatory follicles, this production is stimulated by LH *in vitro* (Etches *et al.*, 1983). It has a direct role in ovulation, specifically in follicular wall breakdown and is also required for albumin secretion. It is also well established that androgens are produced by the small ovarian follicles and all but the largest of the large follicles (Robinson and Etches, 1986). Androgens have been shown to induce ovulation in chickens at supra-physiological levels (see **Figure 1.5.** for illustration). In addition, a passive immunisation with anti-testosterone serum has been shown to block ovulation (Furr and Smith, 1975).

In the final growth phase, as follicles mature, progesterone production by granulosa cells increases and production of androgens and oestradiol by the thecal cells decreases (Etches *et al.*, 1991; Etches and Duke, 1984). Among the large yolky follicles, the granulosa cells of the small follicles (F3-F5) have more FSH receptors and produce more progesterone in response to FSH than those of the larger follicles (F1-F2) (Hammond *et al.*, 1981; Ritzhaup and Bahr, 1987). In contrast, the granulosa cells of the larger follicles are primarily regulated by LH (Hammond *et al.*, 1981). Therefore as the follicles mature, they progress from an FSH to an LH dominated phase; this responsiveness is similar to that observed in mammals. Further work shows that the number of LH receptors in the thecal layers drops suddenly on the day of ovulation in Japanese quail; this may account for the drop in production of androgens and oestrogens by the theca prior to ovulation (Marrone and Hartelendy, 1985).

1.3.3.1. The secretion and circulating level of oestrogen

Most of the oestrogen produced by the ovary of the laying hen are the product of the small follicles of less than 10 mm diameter (Robinson and Etches, 1986), the rest being produced by the larger follicles. A major function of oestrogen is to sensitise the hypothalamic-pituitary axis to the positive feedback effects of

progesterone (Wilson and Sharp, 1976; also see **Figure 1.6**). Other functions include the stimulation of vitellogenesis (Redshaw and Follet, 1972 - see Section 1.3.3); regulation of calcium metabolism (Etches, 1990) and the maintenance of a functional oviduct and secondary sexual characteristics. Whereas progesterone is produced by granulosa cells of the five largest pre-ovulatory follicles, this production is stimulated by LH *in vitro* (Etches *et al.*, 1983). It has a direct role in ovulation, specifically in follicular wall breakdown (Kawashima *et al.*, 1987), and is also required for albumin secretion (Kazemi and Johnson, 1986).

1.3.3.2. Oestrogen regulation of hepatic egg yolk precursor synthesis

The hepatic synthesis of vitellogenin and apo-VLDL-II appear to be completely dependent on oestrogen, since neither protein is normally synthesised in rooster liver (Deeley *et al.*, 1977; Wiskocil *et al.*, 1980). The plasma concentration of each of these increases dramatically after treatment with oestrogen (Wiskocil *et al.*, 1980). The accumulation of triglyceride in the plasma of the laying hen is a result of an oestrogen-induced increase in the rate of synthesis of VLDL in the liver (Kudzma *et al.*, 1975) and decreased rate of clearance of VLDL triglyceride from the circulation (Bacon, 1986).

Oestrogen is also proposed as having an important role in controlling egg weight and that the effect of dietary fats in influencing egg weight is mediated by an influence of the fats on oestrogen metabolism (Whitehead, Bowman and Griffin, 1993). The role of oestrogen in the induction of the preovulatory release of LH has been discovered as the result of a study of the steroid-primed ovariectomised hen (Wilson and Sharp, 1976). The onset of ovarian development is associated with a steady increase in the concentration of plasma LH and oestrogen but not of progesterone (Senior, 1974; Wilson and Sharp, 1975). It can be deduced that the increasing level of plasma oestrogen is due to the steroidogenic effect of LH on the rapidly growing follicles.

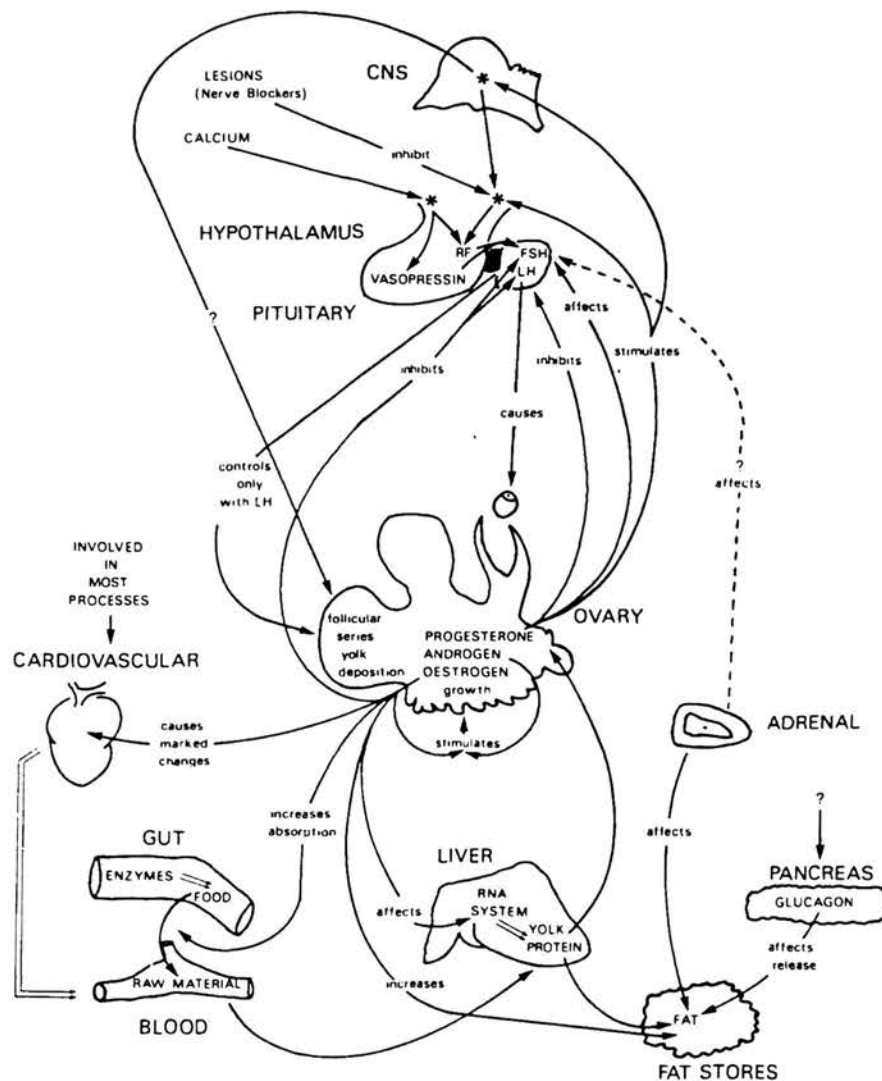


Figure 1.6. Diagram illustrating of some organ interrelationships in ovarian function. Note that not all pathways have been conclusively shown to exist although it is reasonable to assume that they are probably present. FSH= follicle stimulating hormone; LH= luteinising hormone; RF= releasing factors (from Gilbert, 1971).

1.3.4. Hypothalamic and peripheral control of oestrogen

Although the female sex hormones are oestrogens, surprisingly they are not present in high concentrations in either ovarian tissue or blood. The oestrogens in ovarian tissue are oestrone, oestradiol and oestriol (Gilbert, 1971). It is also generally believed that oestrogens act primarily to inhibit LH secretion, paradoxically, there is

evidence that during the initial stages of ovarian development, oestrogens may facilitate the response of the pituitary gland to LHRH (Boney and Cunningham, 1977; also see **Figure 1.6**). However, the fall in the concentration of plasma LH is due to a decrease in the sensitivity of the pituitary gland to LHRH which is probably caused by the negative feedback action of ovarian steroids (William and Sharp, 1977).

The direct action of oestrogen on the pituitary in the chicken is suggested by the presence of an oestrogen receptor (Kawashima *et al.*, 1987). Administration of oestrogen caused a decrease in the binding affinity of the LHRH receptor of the pituitary (Kawashima *et al.*, 1992). It is suggested by Imai *et al.*, (1992) that during each ovulatory cycle a small ovarian follicle about to enter the rapid growth phase can be distinguished by high FSH binding and oestradiol production. A positive feedback response to progesterone, oestrogen or testosterone is lacking in the unprimed ovariectomised hen. (William and Sharp, 1977) also suggests that the preovulatory release of LH is facilitated by the combined actions of oestrogen and progesterone in a two-phase process.

1.4. THERMOREGULATION IN THE DOMESTIC FOWL

1.4.1. Physiological thermoregulation

The physiological mechanisms utilised by the domestic fowl in regulating the deep body temperature indicate that the control system is organised in the same way as that in mammals. The deep body temperature of the common domestic fowl and other domestic avian species range from 41.2 to 42.2°C in contrast to 36.4 to 39°C for domesticated mammals (Whittow *et al.*, 1964). The estimates of average body temperature vary although a value of 41.9°C has been suggested (Smith and Oliver, 1971). In well-fed chickens that are neither dissipating heat to nor gaining heat from the environment, the upper limit of the circadian rhythm is reported as 41.5°C (Etches *et al.*, 1995). The proposed basis of regulation of body temperature is the

temperature difference between a reference signal (T_{ref}) and the actual controlled thermal inputs (T_{in}). Changes in the thermal environment are sensed by thermoreceptors at numerous points throughout the body and fed to the comparator, where the temperature difference ($T_{ref} - T_{in}$) yields an “error” signal which drives the thermo-regulatory actuators (effectors). This activation of control elements adjusts metabolic heat production (MHP) by affecting shivering or heat loss (HL) mechanisms by affecting panting, vasomotion, piloerection, and behavioural responses or both MHP and HL to reduce the temperature difference ($T_{ref} - T_{in}$). See also **Figure 1.7** for details of the thermoregulatory scheme.

In this diagram, body temperature is approximated as a constant that is maintained over a wide range of environmental temperatures, indicated as the zone of normothermia. The lower critical temperature [a] is the minimum environmental temp which is compatible with life. At the upper end of the *zone of normothermia*, body temperature increases [h] in the *zone of hyperthermia* until the critical thermal maximum [g]. This diagram also indicates that increased body temperature is one of the useful heat stress signs. Within the *zone of least thermoregulatory effort* [ZLTE], MHP is at a minimum. Sensible heat loss is relatively constant because physiological and behavioural responses limit the escape of heat throughout this range of environmental temperatures, and EHL (evaporative heat loss) is limited to that occurring as a by product of normal respiration and exposure of non-insulated areas of the body. MHP increases as environmental temperature declines below the *zone of minimum metabolism* (ZMM) and ZLTE [b] to provide energy to maintain the body temperature, and increases above the ZMM [d] to provide energy for panting (this situation may occur in the 'fasting metabolism'). Sensible heat transfer (designated as SHL in **Figure 1.7**), which is accumulative heat transfer from the bird by radiation, conduction and convection, is negative when the environment is colder than the bird and positive when the bird is colder than the environment [e] (Hillman *et al.*, 1985).

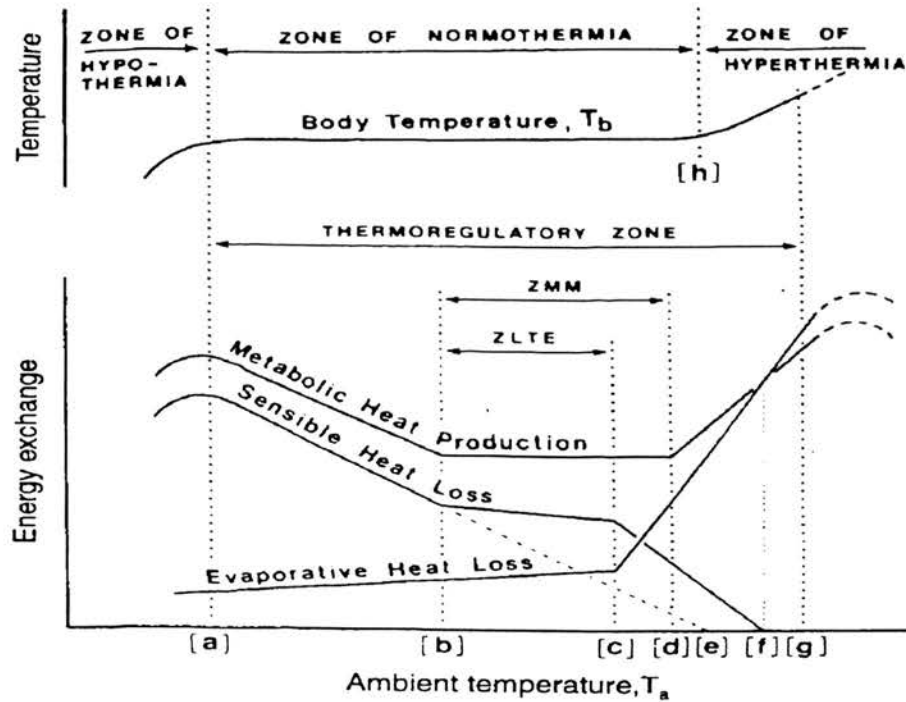


Figure 1.7. Generalised schematic diagram illustrating T_b (body temperature) and partitioning of energy exchange through a wide range of T_a (ambient temperature). The zones and points are: ZMM, zone of minimum metabolism; ZLTE, zone of least thermoregulatory effort; point [a], lower critical temperature; point [b], critical temperature; point [c], temperature at which intense evaporative heat loss begins; point [d], upper critical temperature; point [e], T_a which normothermic T_b ; point [f], where sensible heat loss is zero because MHP (metabolic heat production; in this case as a 'fasting' MHP) equals EHL (evaporative heat loss); point [g], critical thermal maximum; point [h], point of incipient hyperthermia (from Hillman *et al.*, 1985).

Heat can be transferred from the surface of the bird to the environment by sensible heat loss, *i.e.* radiation, convection, conduction and also by evaporative means. While sensible heat can be transferred both to and from the body, evaporative heat transfer can only occur away from the body. Air movement can reduce the degree of heat stress in birds exposed to high ambient temperatures. Various mechanisms exist for birds to protect themselves against the effects of heat; *e.g.* behavioural mechanisms such as reducing activity, reducing body insulation in the short term by raising the wings to expose unfeathered areas, increasing evaporation by an increase in respiration rate, and lowering heat production (Bianca, 1968).

1.4.1.1. Metabolic heat production

Deep body or core temperature is maintained within a narrow range by a high but regulated rate of heat production accompanied by controlled heat loss. The maintenance of a homeothermic internal environment therefore depends upon a dynamic equilibrium between heat production and heat loss, *i.e.* **heat production = heat loss + heat storage**. The maintenance of deep body temperature for long periods is dependent on balancing heat production on the one hand with heat loss and heat storage on the other. Thus deep body temperature is a function of heat production, heat loss and heat storage (Hillman *et al.*, 1985).

Heat is produced by oxidation processes in the active protoplasm of the body and is derived from the breakdown of carbohydrates, fats and proteins. The most important forms of heat production is basal heat production for maintaining essential body processes, like central nerve system (CNS) function, deep body temperature and cardio-respiratory activities (Akester, 1984). Fowls can reduce productive processes and muscular activity and to a more limited extent digestive heat production by a reduction in food intake, but normally they can not reduce basal heat production as minimal body processes must be maintained (Payne, 1990).

1.4.1.2. The control mechanisms of thermoregulation by the hypothalamus

A heat-loss centre may be situated in the anterior hypothalamus, whilst the heat production centre is proposed in the lateral hypothalamus (Dawson and Hudson, 1970). The heat loss centre is activated by the rise in the temperature of the blood bathing its cells. When the blood is cooled, the heat conserving area is stimulated and the body heat is retained, or more heat is produced. The reverse takes place when the blood is warmed. The heat loss centre is also activated by reflexes from the thousands of receptors in the skin (Amakiri and Heath, 1985).

The hypothalamus is considered to perform the role of overall co-ordination and the highest precision in temperature regulation is obtained when the hypothalamus is intact. Each of the multiple effectors (actuators) may be driven by each of the controllers. It is assumed that the controller system with its multiple inputs influences behaviour (Simon, 1974). Thermal inputs into the thermoregulatory system originate from the spinal cord, midbrain, abdomen, and the skin. Behavioural and physiological responses are involved in temperature regulation; the basis of regulation is the temperature difference between a reference signal and the actual controlled thermal inputs (Hillman *et al.*, 1985).

1.4.1.3. Heat loss mechanisms

The main site of evaporative heat loss in birds is the respiratory system and to a lesser extent the skin. The majority heat loss under high environmental temperatures is through evaporative heat loss. This is because the temperature difference between the body surface and the environment is small, under these circumstances the evaporative heat loss is the only avenue which does not depend on temperature differences (Hsia, 1990). The rate of heat dissipation depends on the surface area of the animal, the vapour pressure of the surrounding air (humidity), the rate of air movement surrounding the animal and the extent of respiratory activity (Bligh *et al.*, 1976) despite the absence of sweat glands and the covering of the body in contour feathers.

All body heat produced must be dissipated either by non-evaporative channels (convection, conduction and radiation) or by evaporative channels (moisture vaporisation from the skin and the respiratory tract). Under normothermal condition the greater part of the total heat production is lost as sensible heat or by non-evaporative channels. In the adult hen this constitutes about 75% of the total heat loss, although it may reach up to 90% (Freeman, 1971). Dry heat loss is enhanced by increasing blood flow to the skin, particularly the legs and feet, and by keeping these structures and the whole body if that is possible in shade and in a flow of air (Phillips *et al.*, 1985; Hillman *et al.*, 1985).

Evaporative heat loss (EHL) also occurs in the respiratory process due to evaporation of water, mainly from the upper respiratory tract. In the case of a panting bird, a significant proportion of the total heat loss (THL) is by respiratory evaporative means at high T_a . As the air passes over the wet surfaces of the respiratory tract, the air becomes saturated near body temperature (T_b), although during exhalation it is likely that some heat is lost back to the upper respiratory tract and some water vapour is condensed.

1.5. PHYSIOLOGICAL RESPONSES TO HEAT STRESS

The physiological responses to high environmental temperature in birds involve the functional integration of several organs to meet the metabolic needs of birds that are trying to dissipate heat and maintain homeostasis. In laying hens, the reduction in the plasma concentrations of bicarbonate, due to respiratory alkalosis, compromises egg shell formation by limiting the availability of the anion required during formation of CaCO_3 crystals in the shell (Mongin, 1968). An initial increase in the temperature of peripheral tissues and subsequently in core body is produced when birds are exposed to high ambient temperature (Thornton, 1962; Boone and Hughes, 1971; Wang *et al.*, 1989). Increased body temperature is an essential prerequisite to acclimatisation, however it is not accompanied by increased heat loss. Sykes and Fataftah (1986), reported that acclimatisation is not accompanied by an increase in evaporative heat loss, a situation opposite to that found in man and in other sweating animals. Acclimatisation might also reflect the degree of physiological adaptation of the animal to a particular environment. The greater the extent of adaptation, the better the animal will be able to survive or to reproduce itself so that its biological characteristics may persist.

It is well known that in many cases animal production is reduced by stress imposed on the animal by environmental, nutritional, pathological, and other factors. Fraser *et al.*, (1975) proposes that an animal is said to be in "a state of stress" if it is

required to make abnormal or extreme adjustments to its physiology or behaviour to cope with adverse aspects of its environment and management. The concept of "stress" still eludes satisfactory definition and changes from situation to situation and from user to user. In a biological dimension "stress" evokes a combination of physiological and behavioural adaptations which constitute a "stress response" to aversive stimulus or stimuli which present a challenge to normal homeostasis. Unfortunately this definition is far too simple in view of the complexity of the range of potential stressors to which animals are often exposed to in the real world.

1.5.1. Stress and heat stress

Although many workers have provided a description of the subjective term "stress", the common denominator is the lack of specificity of the conditions believed to be stressful. Stress is more often produced by unpleasant rather than pleasant stimuli which implicates a complex network of psycho-physiological responses, which can be grouped together in what Pasternac and Talajic (1991), called the 'defence reaction' which allows the animal to face a threatening situation or run; thus energy becomes available for '**fight or flight**'. The environmental conditions should, however, be defined by heat load rather than temperature alone. **Figure 1.8** shows the complex relations between dry bulb temperature, wet bulb temperature, equivalent temperature, vapour pressure (water vapour density) and dew point.

Heat stress is a physiological stress induced by ambient temperatures above the upper critical temperature of the thermoneutral zone (see **Figure 1.6**). It is the result of an imbalance between high environmental temperature, heat production and heat loss in the animal. Meltzer (1984) defined "heat stress" as a stress syndrome that appears at high ambient temperatures whether or not the physiological responses prevent a substantial rise in body temperature. A decrease in the performance of chickens housed in hot and humid environments is manifested as reduced food intake to reduce heat generated from the body metabolism, reduced growth rate and reduced food conversion efficiency. If an animal can reduce its heat production and increase

its heat loss under a high environmental temperature then the detrimental effects of heat stress can be reduced to a minimum (Hsia, 1990). However, this balance is easily broken. If an animal cannot reduce its heat production nor increase its heat loss under a high environmental temperature then the animal suffers from heat stress.

1.5.2. Metabolic responses to heat stress

When the birds are exposed to high thermal loads or changes induced in thermoregulatory activity, the circulatory and respiratory systems, body temperature, blood chemistry and endocrine and neuroendocrine activities bring about a wide range of metabolic responses throughout the animal. These will result in alterations in the circulatory profiles of a number of metabolites and intracellular constituents including enzymes. Indeed, various physiological or pathophysiological stressors are known to produce elevation in the plasma levels of intracellular enzymes.

The most evident thermoregulatory behaviour of birds is migration to warmer or cooler areas. Some of the more obvious responses of birds to intense desert heat are to soar at altitudes where air temperature and heat are less than at ground level, or to seek shade, or to reduce activity in the hottest part of the day (Dawson and Hudson, 1970). However these mechanisms have been lost in domestic fowl since they have lost the ability to fly. But some other activities remain like keeping the wings held away from the body and elevation of the scapular feathers. Both these responses facilitate convective heat loss to the air. Chickens also splash water over their combs and wattles, which are cooled by evaporation of water. In cool environments chickens reduce their surface area, and hence heat loss by hunching (Whittow, 1976).

1.5.2.1. Panting and its energetic cost

During exercise in hot environments in domestic cocks, deep panting appears to account for a 12% increase in MHP over exercise alone (Brackenbury and Avery, 1980). On the other hand, an increase in the energetic cost of panting was not observed in the ostrich (*Struthio camelus*) even up to ambient temperature (T_a) as high as 52°C, although the respiration rate did not increase above 45 to 50 breaths per minute. In resting fowl an increase in MHP is not usually observed at T_a between 35 and 40°C, even though the respiration rate increases substantially from about 30 to about 150 breaths per minute (Romijn and Vreugdenhil, 1969). The true 'cost of panting' requires further study. Only using changes in MHP to assess the cost of panting may not be accurate because, as is suggested for mammals, a real increase in metabolic demands by the muscles involving panting may be offset by decreased metabolic demands of other tissues (Weathers and Schoenbaechler, 1976), which results in a little net change in overall MHP.

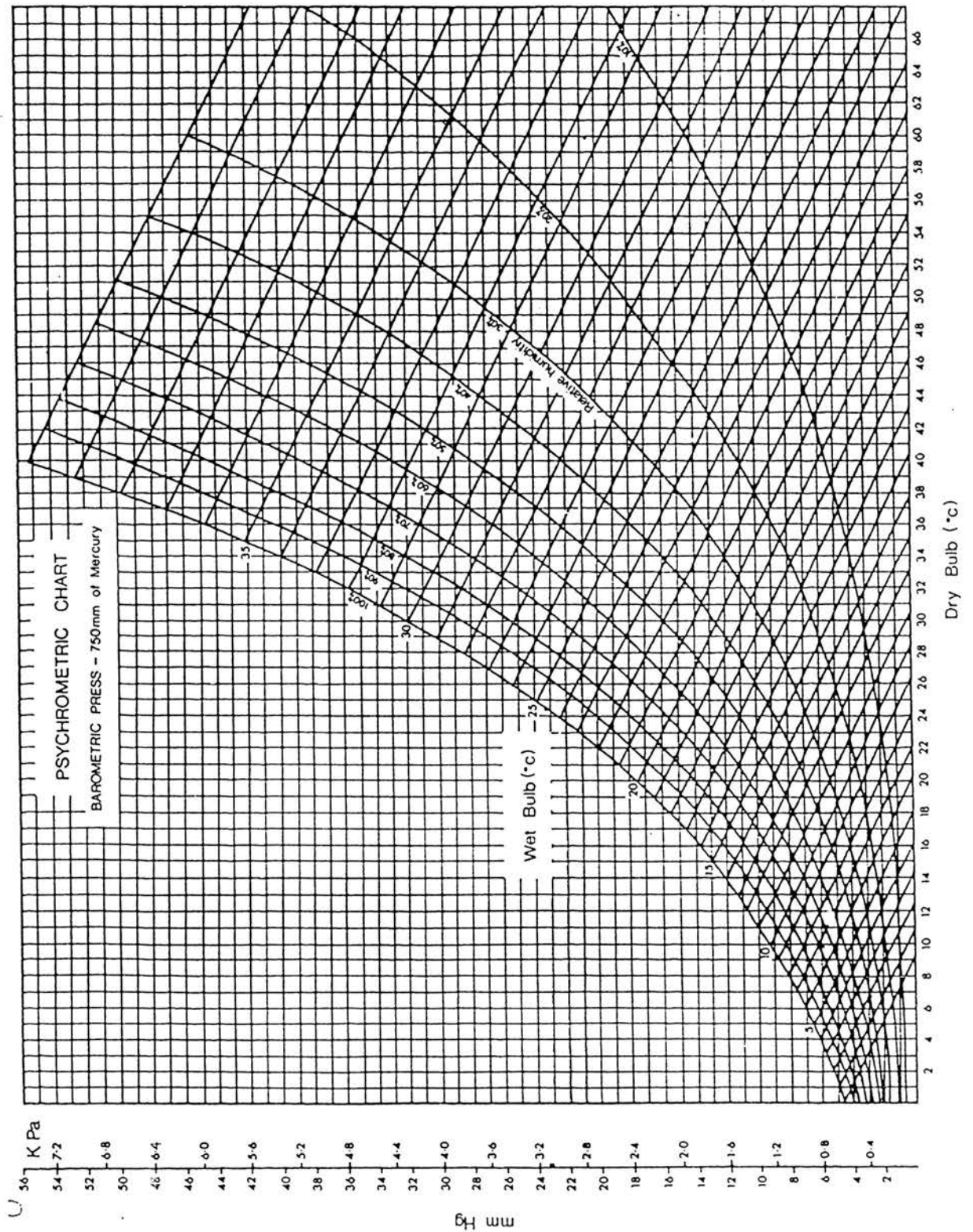


Figure 1.8. The hydrometric chart which shows the real heat loads at different dry bulb temperatures on several water vapour densities. Notice the wet bulb temperatures in the middle of the chart which form the relative humidity.

1.5.2.2. Heat acclimatisation

Acclimatisation may be defined as the long-term adaptive physiological adjustments which result in an increased tolerance to continuous or repeated exposure to complex climatic stressors produced normally under field conditions. Acclimatisation may also reflect the degree of physiological adaptation of the animal to a particular environment. The greater the extent of adaptation, the better the animal will be able to survive or to reproduce itself so that its biological characteristics may persist (Hafez, 1968).

Alterations in MHP following heat acclimatisation are less pronounced than are the alteration of MHP to cold acclimatisation (MacLeod and Mitchell, 1989). In adult fowl, the initial response to heat exposure is reduced heat production (El-Hadi and Sykes, 1980; MacLeod and Hocking 1993; MacLeod *et al.*, 1993). Fasting MHP also declines in heat-acclimated fowl (Davis *et al.*, 1972; Klandorf, Sharp and MacLeod, 1981), which may be due to a drop in thyroid activity (Shafie *et al.*, 1979; MacLeod and Mitchell 1989) or because body weight and egg production drop in layers. Long-term adaptation to heat appears not to be due to increased efficiency of respiratory EHL but rather appears to be due to enhanced SHL by increasing shell conductance by increased blood flow to the non-feathered extremities.

Acclimatisation is possible in the domestic hen allowing it to survive in high ambient temperatures. Sykes and Fataftah (1986a), demonstrated that acclimatisation allowed laying hens to survive during intermittent exposure to a hot, dry climate that initially would have been lethal for them. The increased heat tolerance was reflected in lower body temperatures, higher panting rates and decreased evaporative water loss. Strain differences in the response to heat stress were also suggested by these authors, although it was not concluded whether these were solely a reflection of body size and metabolic rate, or some other genetically determined character.

1.5.2.3. Factors which affect evaporative heat loss

Skin permeability and plumage thickness are two important factors influencing cutaneous water loss. As environmental temperature rises the temperature gradient between the bird and its environment decreases with a subsequent decrease in sensible heat loss. Respiratory rate increases until the bird begins to pant. The onset of panting in resting birds may be abrupt or gradually increase from the normal pattern of ventilation (Bottje and Harrison, 1985a). Heat loss by evaporation can occur through the skin, as cutaneous evaporation, and through the respiratory tract as respiratory evaporation. As humidity increases at high environmental temperature, heat loss from the chicken's respiratory tract in the latent form becomes progressively more important. The birds have to be panting more severely to lose the same amount of heat as those at lower relative humidity. At a fixed temperature, evaporation decreases with increasing air humidity and at a relative humidity of 100% when the air is saturated, evaporation normally ceases, i.e. if the air and surface temperatures are identical (Richards, 1976).

The rate of respiratory EHL is dependent on mean density of air, ventilation rate of respiratory air, latent heat of vaporisation of water at skin temperature, difference between humidity ratio of expired air and humidity ratio of inspired (ambient) air, and latent heat of vaporisation of water at mean the temperature of the surface of the respiratory tract. The water vapour pressure of the atmosphere surrounding a bird affects its rate of total evaporative heat loss (EWL) as shown by the works of Van Kampen (1974) and Richards (1976) (Table 1.1). The maximum cutaneous water lost measured by these authors in the fowl was 1.85 mg H₂O/cm²/hr at 40°C ambient temperature. Another important factor is the difference in water vapour pressure between the evaporative surface and the surrounding air, and this can be affected by the air circulation or ventilation. Such convection will remove humid air and maintain a greater vapour pressure difference across a thinner layer of boundary air. Panting and gular flutter cause the necessary convection across the respiratory surfaces (Phillips *et al.*, 1985).

Table 1.1. Evaporative heat loss (EHL) and water loss (EWL) at various relative humidities.

	20°C	30°C
Total EHL	13.2% MHP at 59% RH	16.5% MHP at 41% RH
Van Kampen (1974)	9.8% MHP at 87% RH	3.3 % MHP at 85% RH
Total EWL	1.5 mg/g/hr at 47% RH	N/A.
Richards (1976)	0.5 mg/g/hr at 100% RH	

As environmental temperature rises the temperature gradient between the bird and its environment decreases with a subsequent decrease in sensible heat loss. The bird comes to rely increasingly on the evaporative heat-dissipating mechanisms in order to maintain its body temperature within the normal range. Respiratory rate increases until the bird begins to pant. It has been suggested that the estimated panting threshold is variable and may be reached at body temperatures between 41-44°C (Maskrey, 1984; Bottje and Harrison, 1985).

1.5.3. Respiratory changes during heat stress

The respiratory system plays a very important role in maintaining homeostasis in heat stressed birds. One of the first signs of heat stress in birds is an increase in the respiratory rate which is related to an increment in body temperature. This increased rate will lead the bird to pant which is the main mechanism of latent heat dissipation. Thermal panting can induce a complex train of events in which pulmonary gas exchange, blood gas transport, tissue gas exchange, cellular metabolism and acid-base balance may each be affected (Kazemi and Johnson, 1986).

The increased respiratory rate, as thermal polypnea, during heat stress will reduce blood carbon dioxide partial pressure (P_{CO_2}) and H^+ ion concentration producing an acid-base imbalance, called respiratory alkalosis (Maskrey, 1984).

Respiratory alkalosis in hens can be developed within 60 min after the onset of an acute thermal challenge (Bottje and Harrison, 1985). Respiratory alkalosis is caused by decrease in P_{CO_2} which increases the HCO_3^-/P_{CO_2} ratio and thus elevates the pH. A decrease in P_{CO_2} is caused by hyperventilation, for example, at high altitude. Renal compensation occurs by an increased excretion of bicarbonate, thus returning the HCO_3^-/P_{CO_2} ratio back toward normal (Ait-Boulahsen *et al.*, 1989). An increase in HCO_3^- raises the HCO_3^-/P_{CO_2} ratio and, thus, the pH. Excessive ingestion of alkalis and losses of acid gastric secretions are also causes of alkalosis. Respiratory compensation sometimes occurs by a reduction in alveolar ventilation that raises the P_{CO_2} . However, respiratory compensation in metabolic alkalosis is often small and may be absent. Base excess is increased; mixed respiratory and metabolic disturbances often occur, and it may be then difficult to unravel the correct sequence of events (Odom *et al.*, 1986).

Thermal panting may be accompanied by increased oxygen demands and increased heat production as a result of the extra work of the respiratory muscles. Additionally to the reduction in P_{CO_2} , severe heat stress may be associated with an increase in blood lactate levels (Marder and Arad, 1989). In a panting hyperthermic bird, the inspired air volume may be up to six times normothermic values.

1.5.4. Blood flow and circulatory changes during heat stress

The cardiovascular system plays an essential part in the responses to temperature changes in the bird's environment. Since birds do not possess sweat glands, panting is the main avenue for heat dissipation, aside from behavioural thermoregulation. Typically, ventilation increase in heat-stressed birds, but this increase on arterial blood gasses differs between different bird species. While the heat-stressed bird is panting, blood flow to the upper respiratory tract is increased and the rate of respiration is increased 10 to 20 times. As a result of such increases, considerable quantities of water are evaporated from the mucus membranes lining the

upper respiratory tract. In order to provide water for evaporation, blood flow to the region is more than doubled, whilst blood to some other parts of the body is reduced (Akester, 1984). Under less extreme conditions, loss of surplus heat occurs without the need for evaporative cooling, by increasing the blood flow to, and thus the heat loss from, those parts of the body which are not feathered. It is generally agreed that peripheral circulation is a modifier of heat transfer to the environment. Dilation of the surface vessels during heat stress allows greater flow of warm blood to exposed parts of the body and thus more heat can be dissipated by convection and radiation mechanisms (Richards, 1976).

In heat stressed birds blood pressure declines, the fall becoming precipitous at body temperatures above 45°C; also there is a reduction in peripheral resistance resulting from dilation of the surface vessels, and in spite of increased cardiac output a reduction in blood pressure is observed. In the terminal stages of hyperthermia cardiac output also decreases. Thus a contributing cause of death in the bird in extremely high temperatures is circulatory failure (Hafez, 1968). In addition, Harrison and Biellier (1969) reported that when birds were moved from 21°C to 35°C environmental temperature the heart rate and blood pressure decreased slowly and respiratory rate and body temperature increased. They concluded that a change in vascular capacity affects blood pressure more than change in heart rate. However, the changes in heart rate during heat stress are not well established and sometimes are contradictory.

1.5.5. Egg formation and production and their responses to heat stress

Egg production in the laying hen is a complex physiological process central to which is the regulation of oocyte development and yolk production. The egg yolk production is influenced by a variety of biological and environmental factors, such as genetic strain of the bird, geographical influences and composition of the feeding pellet (Nemecz and Mennear, 1985). The yolk components are mostly derived from

the blood plasma and the major precursors are vitellogenin and a specialised type of triglyceride-rich lipoprotein. Although the synthesis of yolk is regulated specifically by oestrogen the overall process of vitellogenesis depends upon the gonadotrophins. In addition, FSH and LH not only stimulate endogenous follicular oestrogen synthesis and secretion, but also regulate the uptake of yolk proteins (Follet and Redshaw, 1974).

The hen's egg comprises of three constituents *i.e.* the vitellus or yolk, the most important thing, the white or albumen, and the shell. They are synthesised within different regions of the reproductive tract. The yolk precursors are synthesised in the liver and develop progressively in the ovary, whilst the white and the shell are synthesised in the magnum and uterus respectively (**Table 1.1.2**). In the laying hen the ovary has the appearance of a cluster of follicles, a few of which are in various stages of development and approximately a hundred are small. The accumulation of yolk within the follicles, or vitellogenesis, consists of three successive phases (Larbier and Leclercq, 1994):

- **initial phase**, called slow growth, commencing at hatching. The ovules of an initial diameter of 10 to 20 X 10⁻⁶m grow slowly over 4 to 5 months to sphere with a diameter of 1 mm. Their contents are essentially lipidic in nature.
- **intermediary phase** lasting between 6 and 8 weeks which only concerns a limited number of ovules, being those destined become the yolk of eggs. The diameter increases beyond 4 mm and the contents become enriched with protein giving a whitish aspect (white vitellus).
- **the major phase of growth** where the development is rapid and lasts for between 8-10 days in preparation for the earliest eggs immediately before the laying period. The 6 to 8 relevant follicles enlarge to a weight of 15 to 20g from ones of 200mg. However, they are not all exactly the same stage of development and do not have the same weight. They are laid according to their size, with the largest being the first and the smallest coming several days after, once it has achieved or passed the weight of the previous one.

It is well established that heat stress decreases egg production in laying hens. The reduced egg production is suggested to be at least partly influenced by the ovulatory hormones. In the hen, heat stress reduces serum LH levels, hypothalamic content of LHRH and the preovulatory surges of plasma LH and progesterone (Donoghue *et al.*, 1989; Novero *et al.*, 1991). Since the preovulatory surges of LH and progesterone are controlled in a positive feedback loop (Furr *et al.*, 1973; Etches and Cunningham, 1976; Wilson and Sharp, 1976; see also **Figure 1.6**) and since both hormone levels are depressed concomitantly (Novero *et al.*, 1991), it is difficult to identify the site (or sites?) of action of heat stress. However, the hypothalamus could be a primary target for heat stress because it receives both neural and endocrine inputs that could be translated into general inhibition of the reproductive system.

Table 1.2. The chronology of different stages of formation of the hen's egg.

	Duration	Anatomical position
Formation of the oocytes with yolk deposits	from hatching until ovulation (more than 20 weeks)	Ovary
Ovulation		Infundibulum
Deposition of white	3 h 30 mins	Magnum
Membranes	1 h 20 mins	Isthmus
Shell calcification	21 h	Uterus
Oviposition	-	Cloaca

(From Larbier and Leclercq, 1994)

The blood flow from the body core to the periphery plays a significant role in the transfer of heat from deep body tissues to the peripheral tissues that are capable of dissipating heat to the environment. For example, blood flow through the comb, wattles and shanks is increased due to peripheral vasodilatation and excess heat is dissipated to the surrounding air (Whittow *et al.*, 1964). During heat stress, the

cardiovascular system distributes blood to functions related to thermoregulation, giving only secondary importance to other functions such as those related to the exchange of respiratory gases (Darre and Harisson, 1981) and to the viscera the blood flow to which can be reduced by 44% (Bottje and Harisson, 1984). This may also contribute to the reduced supply of yolk precursors to the developing oocytes.

It is also well recognised that changes occur in the neuroendocrine system due to high environmental temperatures, especially those affecting the hypothalamus and the pituitary glands. These changes may result in less production of gonadotrophins and steroid hormones. This is particularly important in the case of the laying hen considering that the endocrine system regulates the synthesis of egg yolk precursors and probably its deposition in the ovarian follicle (Miller and Sunde, 1975; Redshaw and Follett, 1972).

Decreased egg production could be due to hormonal changes and/or disturbances in the hen's egg production cyclic process. In the laying process there are a lot of demands upon the availability of protein, lipids and calcium as well as energy intake. Therefore, there are some possible physiological mechanisms which may be responsible for the decrease in egg yolk production in the laying hen during exposure to heat stress. Synthesis of vitellogenin in the liver may be altered, with or without a decrease of oestrogen concentration in the circulation; or there may be disturbances in the transport of vitellogenin in the blood stream; and/or changes in the level of ovarian uptake by the growing oocytes.

It is well known that heat stress reduces egg production, egg size and egg shell thickness, while improving food efficiency (De-Andrade *et al.*, 1977; Miller and Sunde, 1975; Deaton, McNaughton and Lott, 1982; Deaton, Reece and Lott, 1981; Yamada and Tanaka, 1988), and egg production is more affected by constant high ambient temperatures than by cyclic high temperatures (Payne, 1966; Miller and Sunde, 1975; Yamada and Tanaka, 1988). In addition, Emery, Vohra, and Ernst (1984), concluded that egg weight and shell thickness were significantly reduced by environments cyclic between 15.6 to 37.7°C. They finally suggested that the reduction in these parameters was not due to a significant reduction in feed intake, but

was a direct effect of the heat exposure on the hens. Payne (1966) and De-Andrade *et al.* (1976) have suggested that the detrimental effects of heat stress on egg production could be minimised by increasing energy levels of the diet in order to benefit from the inadequacy of regulation of energy consumption as dietary energy level is changed.

In addition Odom *et al.* (1986) reported that a persistent blood alkalosis was developed in Leghorn hens after the chamber temperature reached 35°C. Accompanying this steady rise in arterial pH there was a decrease in the ionised calcium concentration. The arterial P_{CO_2} exhibited a decline to a low value two hours after the start of the heat exposure. At this point the ionised calcium level was reduced by 15% and there was an increase in pyruvate and lactate.

1.6. REDUCING THE EFFECTS OF HEAT STRESS AND THE ROLE OF VITAMIN E

Various methods have been applied in order to alleviate the effects of heat stress. These have included the use of high nutrient density diets, carbonated drinking water, incorporation of fat into the diet, administration of vitamin C (Perek and Kendler, 1962, 1963) and more recently supplementation with vitamin E (Utomo *et al.*, 1993; Utomo *et al.*, 1994). However, the results observed so far are not conclusive and sometimes are even contradictory. Some of the methods used in overcoming the detrimental effects of high temperatures, includes control and improvement of the thermal environment; selection of breeds with better heat tolerance; and modification of diet. Where possibilities of improving management conditions are limited, breeding concepts and dietary modifications gain in importance (Ota and McNally, 1963; Deaton, 1983). To reduce the heat increment in heat stressed birds, including reduced energy intake during the day time, reduced protein and amino acid levels, fat feeding, and other nutritional strategies have been applied. Sykes and Fataftah (1980) demonstrated that the addition of maize oil to the diet of

acclimatised laying hens led to a loss of heat tolerance because of the increment in energy intake.

Heat stress may promote oxidative damage to cells through free radical formation. Excessive amounts of active oxygen results in lipid peroxidation and damage to organelles, cells and their membranes. In normal animals there is sufficient endogenous antioxidant capacity to remove active oxygen but this does not occur in stressed animals. There are a number of compounds occurring in nature which exhibit an action characteristic of vitamin E. These substances are known as tocopherols and tocotrienols; α -tocopherol has the highest biological activity (Hennings *et al.*, 1986; Chen, 1992). Incorporation of vitamin E (α -tocopherol) in the diet may help to reduce lipid peroxidation because it interacts with selenium-containing glutathione-peroxidase to prevent the oxidative damage.

1.6.1. Vitamin E and its antioxidant capacity

Natural fats possess a certain degree of resistance to oxidation owing to the presence of compounds termed antioxidants. These prevent the oxidation of unsaturated fats until they themselves have been transformed into inert products. A number of compounds have this antioxidant property, including phenols, quionones, tocopherols, gallic and gallates. The most important naturally occurring antioxidant is vitamin E which protects fats by preferential acceptance of free radicals (Combs *et al.*, 1975).

Vitamin E was discovered by Evans and Bishop in 1922 and was thought to be associated with fecundity. In fact it only has this role in the rat. Vitamin E is a group name which includes a number of closely related active compounds and is characterised by tocopherols. Eight naturally occurring forms of the vitamins are known and these can be divided into two groups according to whether the side chain of the molecule is saturated or unsaturated. The four saturated vitamins are designated α -, β -, γ - and δ -tocopherol and of these the α form is the best known and most biologically active and most widely distributed (McCay, 1985). Although

oxidants which protect vitamin A, carotene, and unsaturated fatty acids as well as maintaining the stability of intracellular membranes in the erythrocyte which contain a high proportion of unsaturated fatty acids (Larbier and Leclercq, 1994).

The β , γ and δ forms have only about 45, 13 and 0.4 per cent of the activity of the α form respectively. The unsaturated forms of the vitamin have been designated α -, β -, γ - and δ -tocotrienol and of these only the α form appears to have any significant vitamin E activity, and then only about 13 percent of its saturated counterpart.

Tissues such as liver and blood absorb more of the administered dose of vitamin E than other organs, but are depleted more rapidly; on the other hand, heart, lung, and adrenals show a much slower turn over rate and still contain tocopherol after many weeks of depletion. The plasma plays an important part in tocopherol transport, the vitamin apparently being partly in the neutral fat fraction but mainly attached to the globulin fraction. Intracellular vitamin E is found predominantly in the mitochondrial and microsomal fractions (Henning *et al.*, 1986). The vitamin E values of foods are often expressed in terms of International Units (IU), one IU of vitamin E being defined as the specific activity of 1 mg of synthetic racemic α -tocopherol acetate (Combs, 1975).

Vitamin E function in the animal mainly as a biological antioxidant; in association with the selenium-containing enzyme glutathione peroxidase, it protects cells against oxidative damage caused by free radicals. Free radicals are formed during cellular metabolism and, as they are capable of damaging cell membranes, enzymes and cell nuclear material, they must be converted into less reactive substances if the animal is to survive. This protection is particularly important in preventing oxidation of polyunsaturated fatty acids which function as primary constituents of subcellular membranes and precursors of prostaglandins. Oxidation of unsaturated fatty acids produces hydroperoxides, which also damage cells and tissues, and produce more lipid free radicals so that prevention of such oxidation is of vital importance in maintaining the health of the living animal.

There are several other mechanisms for animal in protecting itself against oxidative damage. Two of the main methods to prevent the damage are firstly, radicals are scavenged by vitamin E as a first line of defence; and secondly, glutathione peroxidase destroys any peroxides formed before they can damage the cell (McCay, 1985). The two defence mechanisms complement each other (Hoekstra, 1975). Vitamin E also plays an important role in the development and function of the immune system (Chen, 1992). Supplementation of diets with the vitamin provided some protection against infection with pathogenic organisms (Richter *et al.*, 1986).

Vitamin E (α -tocopherol) is involved in several crucial metabolic processes. It mainly acts as an inter-and intracellular antioxidant protecting the unsaturated fatty acids both in the diet and in the cell membrane. The inhibition of lipid peroxidation by antioxidants is as well documented as the biochemical function of vitamin E as an antioxidant (Tappel, 1968; Combs *et al.*, 1975). It is also part of several metabolic pathways and helps to maintain the integrity of blood vessels (Fowler, 1990). The intramembrane antioxidant properties of vitamin E may protect the sarcolemma from lipid peroxidation due to free radical attack and minimise the associated loss of integrity and increased permeability. The unsaturated double bonds of membrane polyunsaturated fatty acids (PUFAs) are inherently unstable and are readily attacked by peroxides and other forms of active oxygen. This process tends to produce a chain reaction and more free radicals and hydroperoxides are produced.

1.6.2. The role of vitamin E in tissue membrane protection

Stress can lead to over production of oxygen free radicals OH^\cdot and O_2^\cdot (Slater, 1984). Free radicals can cause metabolic disturbances and cell injury in a variety of ways. They also cause profound changes in enzyme activity and may also damage cells by lipid peroxidation of PUFAs with direct effects on membrane structure. This is by far the most important damage produced by free radicals in the animal (Slater, 1984). In heat-stressed animals, their normal antioxidant capacity can be

exhausted. Thus free radicals can initiate and propagate peroxidative damage to several cell constituents including PUFAs in cell membranes. Such decomposition leads to the disruption of cell membranes and cause an increased leakage of enzyme such as creatine kinase (CK) (Mitchell and Sandercock, 1995) and pyruvate kinase (PK) from tissue to plasma (Duthie *et al.*, 1989). This damage can be particularly serious in organs like the liver and muscle because of their high metabolic activity (Fowler, 1990).

Vitamin E (α -tocopherol) acts as a scavenger of free radicals and prevents this explosive reaction (Putnam and Comben, 1987) and has also been associated with selenium (Se) in the prevention of lipid peroxidation. Selenium is an integral component of the enzyme glutathione peroxidase (GSH-Px). This enzyme is a key component of the antioxidant system (Martensson *et al.*, 1991) and it has been suggested that both vitamin E and Se have complementary roles in the prevention of oxidative damage (Hoekstra, 1975); however they are not exchangeable. In the absence of adequate Se to form GSH-Px, cells will contain excessive peroxides which will attack unsaturated lipids in spite of the protection of adequate vitamin E (Putnam and Comben, 1987).

A relationship between vitamin E and vitamin C has also been proposed by McCay (1985). It was suggested that ascorbate either has a sparing action on tocopherols by itself acting as an antioxidant or it acts on the tocopheroxyl radical, the oxidised form of tocopherol to remove the oxygen and so regenerate active α -tocopherol. It is also proposed that maintaining an adequate constant supply of vitamins and minerals to the tissues vulnerable to atherosclerosis (*e.g.* the vascular endothelium) may contribute importantly to the protective mechanisms that prevent or reduce blood vessel wall injury. Of particular interests are vitamin E and zinc, which may be able to function as antioxidants and membrane stabiliser (Putnam and Comben, 1987; Henning *et al.*, 1986).

It is therefore necessary to elucidate the important mechanisms involved during the exposure of laying hens to high ambient temperature in particular of egg and yolk production in conjunction with the changes its precursors in the circulation.

This includes necessarily to evaluate the uptake by the oocytes, the role of oestrogen and other physiological responses. Further studies also have been carried out to alleviate the effects of heat stress in the laying hens using supplementation of the diet with vitamin E (α -tocopherol). This is expected as of a possible solution to improve laying performance under heat stress conditions. The study provides important evidence of a possible physiological basis for the changes in yolk and egg production induced by chronic heat stress in laying hens.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

This chapter describes the general materials and procedures common to all experiments. Particular details of each experiment and the modifications of general techniques are provided in the appropriate sections.

2.1. ANIMAL HOUSING AND HUSBANDRY

2.1.1. Experimental birds

The birds for these experiments were layers from a commercial (ISA Poultry Services, Peterborough, UK), laying strain genetically selected for high rate of egg production. The day-old chicks were reared and maintained at the Roslin Institute (Edinburgh) in brooders ($29 \pm 1^\circ\text{C}$). All the day-old-chicks were vaccinated against Marek's disease and received a booster after 3-weeks. At 4-weeks-old they were vaccinated for Newcastle and Gumboro diseases and boosted at 16-weeks of age. Water was available *ad libitum* through drinking nipples. Prior to the laying period the birds were kept individually caged under a 24 h artificial lighting pattern (14L:10D photoperiod; lights on at 06:00-h GMT). The birds used were at a range of ages extending from 32 to 65 weeks from one experiment to another, but the hens used in any one study were of the same age, *i.e.* they had been reared in the same batch. Egg laying records were kept for each hen and only hens which had a good laying record were selected for study. Most hens laid between 4-6 h after the beginning of the light period. On the first day of an experiment birds were transferred to controlled-climate chambers, caged individually and exposed to the specified temperatures and relative humidities. At the end of each

experiment birds were killed by administration of a lethal dose of pentobarbitone sodium (Rhône-Mérieux, Southampton, UK) injected intravenously through the brachial vein.

2.1.2. Controlled climate chambers

Three controlled climate chambers (3.5 x 2.0 x 2.0 m) were used. The chambers were maintained at different climatic conditions which depend on the experimental requirements. Temperature could be controlled within the range -5 to +40°C ($\pm 0.2^\circ\text{C}$) and relative humidity (RH) between 10 and 100% ($\pm 5\%$). The temperatures and relative humidities (RH) of chambers were measured continuously throughout the experiments and recorded every 5 min by a Data Logger (1200 Series Squirrel Meter/Logger, Grant Ltd., UK). The accuracy of the chambers were satisfied in maintaining the temperature and relative humidity as shown in **Figure 2.1**. The data recorded by the Logger were transferred to a PC by 20/20™ programme (Access Technology Ltd., Buckinghamshire, UK).

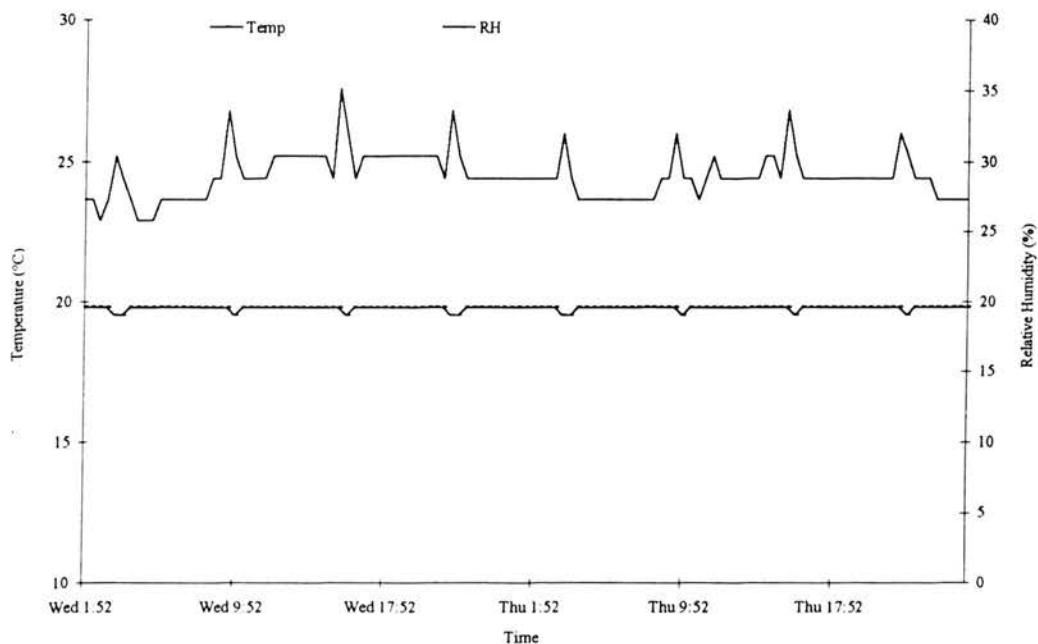


Figure 2.1. Temperature and relative humidity of the chambers can be controlled within the range -5 to +40°C ($\pm 0.2^\circ\text{C}$) and between 10 and 100% ($\pm 5\%$) RH. The temperatures and relative humidities were measured continuously throughout the experiments and recorded every 5 min by a Data Logger.

2.1.3. Battery cages

The battery cages were designed in a three-tier module. Each module had 12 individual cages (48 x 30 x 46 cm) with four cages per tier (**Figure 2.2**). Each cage was equipped with an individual feeder, which allowed measurement of individual food intake, and an automatic nipple-drinker. Each controlled-climate chamber had the same design and lighting regime.

Cage 1	Cage 2	Cage 3	Cage 4	<i>Top tier</i>
Cage 5	Cage 6	Cage 7	Cage 8	<i>Middle tier</i>
Cage 9	Cage 10	Cage 11	Cage 12	<i>Bottom tier</i>

Figure 2.2. Arrangement of cages in a laying hen battery module.

2.1.4. The food and vitamin E supplementation in the diet

The control diet was a standard layer's mash containing 160 g crude protein, 11 MJ metabolisable energy and 35 g calcium/kg (also see **Table 2.1** for details). In some experiments (**Chapter 7**) which used vitamin E supplementation in the diet, the vitamin E diet was made from the control diet supplemented with 500 mg of vitamin E (powder form with 50% activity; absorbed on expanded silica) per kilogram diet. Unless otherwise indicated the food and water were provided *ad libitum*. The vitamin E product used in this trial was the Microvit E 50 ADS[®] (Rhône Poulenc Animal Nutrition) containing 500 mg of DL α -tocopheryl acetate per gram of product.

Table 2.1. Ingredients and calculated nutrient composition of the normal diet.

Raw material	g/kg	Calculated nutrient composition	g/kg
Code: LP			
Barley meal	300.00	Fat	37.70
Grass meal	50.00	Dry matter	881.61
Limestone flour	75.00	Cysteine	2.58
Lysine hydrochloride	0.90	Leucine	10.74
Meat and bone meal	70.00	Methionine	8.79
Methionine	1.10	Threonine	6.20
Mineral mix 5	2.50	Calcium	35.74
Sodium chloride	2.50	Phosphorus available	4.00
Soyabean meal (44% CP)	120.00	Phosphorus total	7.00
Vegetable oil	15.00	TME _N	11.20
Vitamin mix 6 ^{*)}	3.00	Crude fibre	39.02
Wheat meal	360.00	Crude protein	160.42
Total	1000.00		

^{*)} Vitamin mix 6 contains 10 ppm of vitamin E

2.1.5. Deep body temperature and food intake monitoring

Daily food intake for every bird was recorded each morning between 09:00-10:00 h except in the paired and restricted feeding experiments (see **Chapter 5**). The mean values of body weight, rectal temperature and food conversion efficiency for the experimental groups of birds were calculated using Minitab (Minitab Release 10, Minitab Inc., UK). The birds' body temperatures were measured twice a week at the same time that blood samplings were taken so as to minimise the stress of handling. The measurements of deep body temperature were performed within the climate chamber under the appropriate environmental conditions to avoid any change in body temperature. An electronic rectal probe (thermometer model RS-612-849) was used to measure the deep body temperature. Thermometer was inserted approximately 5 cm into hen's rectum to measure the deep body temperature.

2.2. EGG MEASUREMENTS

2.2.1. Egg production

Eggs were collected, individually labelled and weighed on a daily basis. The egg collections were done from 10.00 to 18.00 h. The eggs collected on a particular day were assumed to be the egg production for that group on that day. Any broken eggs collected were regarded as missing data for the egg and yolk weights but they were included in the egg production records. The means of egg and yolk weight were multiplied by the mean of egg production to yield the total egg and yolk production.

2.2.2. Egg and yolk weights

The eggs obtained were weighed as soon as they were laid. After taking the weight the eggs were broken and the albumens were separated from the yolks, using a yolk separator. The egg yolks were placed on a hospital roll or tissue to detach any adhered white materials before they were weighed.

2.2.3. Shell weight and shell thickness

In order to have a constant weight egg shells were dried overnight in an oven at 60°C. On the following day the dried shells, including the very thin layer of adhered membrane, were weighed. Shell thickness was measured by using a micrometer and was done three times at different sites of the shell, *i.e.* at the middle and at two poles of the shell. The figures obtained were then averaged to obtain mean shell thickness. However, the shell thickness measurement, especially at the poles of the shell, was not satisfied as the micrometer could not reach the narrow part of poles. Therefore, the data of this measurement were omitted from the results and discussion.

2.2.4. Pattern of daily yolk production

Yolk proteins are composed of three fractions *i.e.* phosphoproteins (phosvitin, lipovitellin and vitellogenin), triglyceride-rich lipoproteins and livetins (yolk water-soluble proteins). Incorporation of the major yolk precursor VLDL is accomplished by receptor-mediated endocytosis (Griffin *et al.*, 1984).

The basic principle of this method is to incorporate a lipid soluble dye (lipophilic material) into blood circulation through the diet. The dye is taken up and bound by the yolk precursors. The dye is capable of colour-labelling the egg yolk produced and deposited in the ovary on that day. This technique allows the investigation of the amount of yolk deposited in the oocyte on a daily basis as proposed by the methods of Gilbert (1971).

2.2.4.1. Preparation and feeding the dye to the birds

Two lipophilic coloured dyes (Sudan black and Scarlet R [red]) were used to colour the egg yolk materials while they were being deposited in the ovary. The two dyes were put into different capsules for each colour. The capsules were inserted into the bird's oesophageal space through the beak to make sure that the capsule was definitely consumed. The two different coloured the dyes were given on alternate days (red₁-black₂-red₃, etc.).

2.2.4.2. Measurement of daily yolk production

Each day the growing follicles were coloured with the different dyes. To fix the coloured yolks the eggs were boiled and the colours appeared clearly as rings in the sectioned egg yolks. To calculate the amount of yolk mass in each ring the diameters of the rings were measured using a micrometer and taken three times at different sites on a ring. The figures obtained then were averaged to obtain mean ring diameters. The volume was calculated based on the assumption that the rings formed "concentric spheres". Thus the sphere volume could be calculated using the

formula $V = (\pi \times d^3)/6$; where V = volume; $\pi = 3.142$; d = diameter). Daily yolk production was calculated by subtracting a “sphere” volume with the nearest smaller sphere (see **Figure 2.3**).

$$V = V_2^3 - V_1^3 \Rightarrow V = \left(\frac{\pi \times d_2^3}{6} \right) - \left(\frac{\pi \times d_1^3}{6} \right)$$

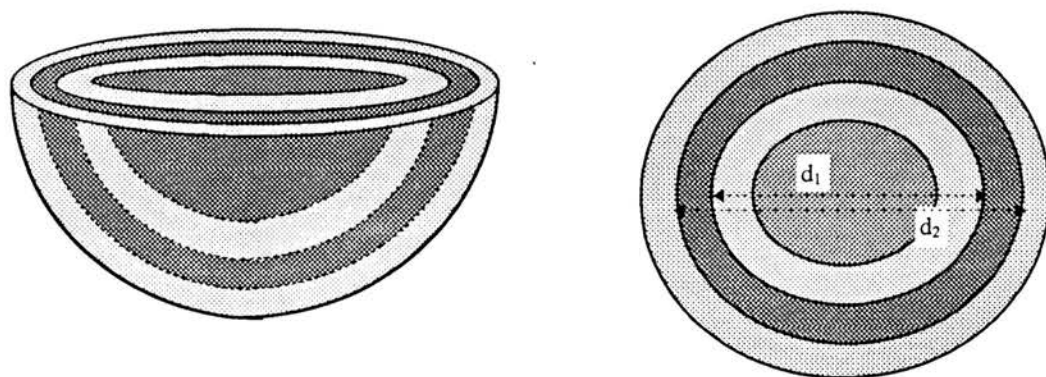


Figure 2.3. The coloured-yolk materials deposited in the oocyte formed concentric spheres which showed the yolk materials uptake by the follicle (the picture was taken from a boiled egg and was not scaled).

2.3. COLLECTION OF BLOOD SAMPLES

Blood samples (about 2.5-3.0 ml) were withdrawn into heparinised (heparin in physiological saline 50 units/ml, Evans Medical Ltd) syringes from the brachial vein and immediately transferred to heparinised tubes (3 ml tube - Teklab). The blood was placed on a roller (Spiramix 5 roller, Jencons (Scientific) Ltd.), and then centrifuged at 1500 g for 10 min (Denley BS 200 centrifuge) to obtain the plasma fraction. Plasma was stored at -20°C for later analysis.

2.4. PLASMA ASSAYS

Protocols for all the kits below were modified for use with an automatic plate reader (Titertek 2, Flow Laboratories UK Ltd.). All Wako products were supplied by Alpha Laboratories UK Ltd.

2.4.1. Determination of plasma total and VLDL-depleted zinc

Plasma vitellogenin was determined by the measurement of the zinc concentration in the plasma; it was measured on the whole and VLDL-depleted plasma by the method of Mitchell and Carlisle (1991) using a commercial kit (Zn-Wako Chemicals GmbH). Each sample was deproteinised by addition of an equal volume (v/v) of 7% trichloroacetic (TCA) and centrifugation at 1500 *g* for 10 min and the supernatant removed for analysis.

Zinc standards were prepared by halving dilution of a 2 µg/ml stock from the kit with distilled water (v/v) to give 1, 0.5 and 0.25 µg/ml. Standard, blank and unknown samples were dispensed in LP4 tubes in a volume of 100 µl; 100 µl of 7% TCA was added and the mixture was vortexed and centrifuged at maximum (4000 *g*) for 10 min. A 40 µl supernatant of each was removed and put into a micro plate. The chromogen solution, a solution which reacts with the sample or mixture and produces colour, was prepared *i.e.* colour reagent A and B mixed 4:1 (v/v) using clean measuring cylinders to avoid zinc contamination. Chromogen solution (see **Table 2.2.** for its constituents) was added into each well in a volume of 200 µl and left for 10 min the absorbance measured at 520nm at room temperature. The stability of the working chromogen solution was 1 week at 2 to 10°C or 2 days at room temperature. Results obtained by this assay correlated well ($r = 0.96$) with those by atomic absorption spectrophotometry. The linearity remains up to 1000 µg/dl (153 µmol/L).

Precipitation of vitellogenin and VLDL. Vitellogenin and triglyceride rich VLDL were selectively precipitated from plasma by dilution with an equal volume (300 ml) of an aqueous solution in a LP4 tube (containing 0.9 % NaCl, 0.2% Dextran-SO₄, 200mM MgCl and 10mM Tris buffer adjusted to pH 7.4 with HCl) as described by Griffin and Mitchell (1984). The dextran sulphate solution (50 ml) contained 0.45g NaCl, 1.00g Dex-SO₄, 2.03g MgCl and 0.06057g TRIS(hydroxymethyl) methylamine. Samples were vortexed and centrifuged at maximum (4000 *g*) for 5 min and 100 ml of supernatant was removed for zinc assay.

Ionised magnesium (Mg^{+}) of the precipitation solution bound large protein electrostatically and reacted and bound by dextran to make higher molecule weights. This allowed vitellogenin and VLDL came to the solution. The results were converted for dilution factor (2x). The VLDL-depleted zinc result was subtracted from the total zinc result to give the level of vitellogenin zinc as follows:

$$\text{Precipitated zinc concentration} = (\text{Total zinc} - \text{VLDL depleted zinc}) \text{ concentration.}$$

Table 2.2. Reagents for the assay of plasma zinc.

Description	Constituents	Concentration (mmol/L)
Colour Reagent A	5-Br-PAPS	0.08
	Bicarbonate buffer pH 9.75	200
	Sodium citrate	170
	Dimethylglyoxime-	4
	Triton X-100	
Colour Reagent B	Salicylaldoxime pH 3.0	29
Deproteinising Reagent	Trichloroacetic acid	370

2.4.2. Determination of plasma triglyceride (VLDL)

All plasma samples were diluted 3-fold with distilled water (1 to 2 v/v) to adjust to the standards curve and the results multiplied by dilution factor (3x). Triglyceride was measured by means of a commercial kit (Triglyceride-N, Wako Chemicals GmbH) using tri-oleins as standard at a maximum concentration of 3 mg/ml. It was a neat standard supplied with the kit. Halving dilutions were made to produce the other two standards (1.5 and 0.75 mg/ml). The colour reagent was prepared by completely dissolving the contents of the colour reagent in one bottle of buffer solution with gentle tilting. The constituents of the colour reagent were listed on **Table 2.3**. The stability of the colour reagent was 4 weeks at 2 to 10°C or 4 days at 20 to 25°C.

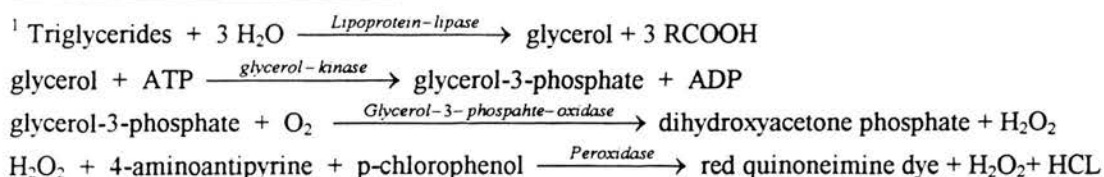
Table 2.3. Reagents for the assay of plasma triglyceride.

Description	Constituents	Concentration
Colour Reagent	Lipoprotein lipase	> 75.000 U/L
	Glycerol kinase	> 500 U/L
	L- α -Glycerol-phosphate-oxidase	> 1.250 U/L
	Peroxidase	> 220 U/L
	ATP	0.09 mmol/L
	4-Aminoantipyrine	0.35 mmol/L
Buffer solution	Good's Buffer	50 mmol/L; pH 7.5
	p-Chlorophenol	5.44 mmol/L200

Zinc standards were prepared by halving dilution of a 2 $\mu\text{g/ml}$ stock from the kit with distilled water (v/v) to give 1, 0.5 and 0.25 $\mu\text{g/ml}$. Standard, blank and unknown samples were pipetted into wells of a micro plate in a volume of 6 μl and 300 μl of colour reagent was added. Absorbencies against colour reagent solution blank read on a plate reader (at 505 nm) at room temperature 30 min after adding the colour reagent ((see **Table 2.4** for summary of the procedure). The dye formed was stable for 1 h at room temperature¹.

Table 2.4. Procedure for the assay of plasma triglyceride.

	Sample	Standard	Blank
Sample	6 μl (Plasma)	6 μl (Standard solution)	6 μl (distilled H ₂ O)
Colour reagent	300 μl	300 μl	300 μl



2.4.3. Determination of plasma inorganic phosphorus

Inorganic phosphorus was determined using a commercial kit (Phosphor B, Wako Chemicals GmbH) using standards in the range 12.5 to 100 µg/ml. Inorganic phosphorus is not used to measure vitellogenin, unlike organic phosphorus which is as protein bound phosphorus with vitellogenin in the plasma, but inorganic phosphorus may be linked to total calcium from bone metabolism for eggshell formation.

A 10 µl of standards, blank and samples were pipetted into each well (see **Table 2.5.** for the illustration). Colour reagent (250 µl) were added and thoroughly mixed into each well and allowed to stand at room temperature for 15 min before being read on a plate reader at 690 nm.² The dye formed was stable for 4 h at room temperature. A standard curve was again constructed by dilution of standards provided with the kit. Coefficient variation within assay was 2.9% and between assay variation was 5.7%.

Table 2.5. Procedure for the assay of plasma inorganic phosphorus.

	Sample	Standard	Blank
Sample	10 µl (Plasma)	10 µl (Standard solution)	10 µl (distilled H ₂ O)
Colour reagent	250 µl	250 µl	250 µl

2.4.4. Determination of plasma total calcium

Plasma total calcium was determined using a commercially available kit (Wako-Calcium C). The kit was modified for use with avian body fluids and for absorbance determination on an automatic plate reader. Samples were free of cation chemicals and contamination with extraneous calcium (blood samples were treated with heparin not oxalate or EDTA) and all glassware washed in dilute HCl and rinsed

² phosphate + ammonium molybdate → ammonium phosphomolybdate
 ammonium phosphomolybdate + aminophtholsulphonic acid → heteropolymolybdenum (blue colour)

in ion free water (not tap water). In general samples may be analysed neat but for laying hen plasma must be diluted as it contains high calcium concentration in the circulation. The plasmas were diluted two-fold (100 μ l of plasma added to 100 μ l of PBS pH 7.4 and mixed). Any precipitates were removed by centrifugation at 2500 *g*. Two standards were supplied with the kit, *i.e.*: standard (STD) 1 and STD 2 containing 2.5 and 5.0 mM calcium respectively. STD 2 was used as the top standard (neat) and STD 1 with halving dilutions with ion free water was used to give STD 3 to STD 5 at 1.25, 0.625 and 0.312 mM. The principle of the assay was that alkali earth metals reacted with ortho-cresolphthalein complexon (OCPC) to form a coloured complex which was red to purple.³ An estimate of assay variation was calculated by running an aliquot of pooled plasma on every plate. Within assay variation was 3.1% and between assay variation was 6.9%.

Assay procedure. Blanks, standards or samples were added to the appropriate wells on a micro plate in a volume of 2 μ l and then 200 μ l of assay buffer were added and mixed in. 20 μ l of the colour reagent was added and mixed thoroughly and allowed to stand at room temperature for 15 min. The absorbance was measured on a plate reader at a wavelength of 560 nm. The colour developed in this assay procedure was stable for up to 2 h. Results were calculated with correction for dilutions.

2.4.5. Determination of ionised calcium and blood pH

In blood circulation calcium exists in its two different form, *i.e.* ionised or 'free' calcium and the second is 'bound' calcium. Ionised calcium is regulated to remain within a tightly defined range because it affects a wide range of biological processes. The 'bound' form is attached to plasma proteins, mainly albumin, and citrate. In mammals about 50% of the total blood calcium is ionised. In birds, the picture is slightly more complex. Egg-laying females need to transport large amounts

³ a purple calcium-cresolphthalein complexon complex is formed by calcium and cresolphthalein complexone (OCPC) in ethanolamine-borate buffer. 8-Hydroxyquinoline in the reaction mixture makes the test specific for calcium.

of calcium to the shell gland for synthesis of egg shell. Vitellogenin binds calcium and allows it to be transported in the blood (Guyer *et al.*, 1980) but does not transport calcium to the shell gland. Therefore, the plasma level of total calcium increases with the attainment of sexual maturity. The concentration of ionised calcium also varies cyclically in the reproductively active female. Ionised calcium is highest when the shell gland is empty and then declines following the entry of the egg into the shell gland and reaches a minimum level 16 hours before the next oviposition (Parsons and Combs, 1981). The main source of calcium is from the diet although the availability in the circulation might also be supplied from medullary bone (Miller and Sunde, 1975; Etches, 1987).

The ionised calcium concentration and blood pH in whole blood were measured using an automatic calcium/pH analyser (Ca^{2+} /pH Analyser, Ciba-Corning 634). Blood samples were taken and put into tubes containing 50 units lithium (Li)-heparin. It is suggested that Li-heparin will only bind calcium at a concentration higher than 50 units/ml (Utomo and Mitchell, unpublished data; Rennie, 1994). Red blood cells of chicken differ from those of mammals in that they are nucleated and have mitochondria. Therefore, during the determination of ionised calcium and blood pH the tubes were always capped and kept on ice to reduce respiration by erythrocytes, which consumes O_2 and produces CO_2 , to stabilise the blood pH which may alter the concentration of free calcium. Measurements were taken within 2 min of blood collection.

2.4.6. Validation of techniques for measurement of total and ionised calcium

The purpose of the validation of the techniques was to determine the accuracy of the measurement of calcium and to cross calibrate the measurements of total and ionised calcium using the colorimetric and ionised calcium electrode methods described in **Sections 2.4.4 and 2.4.5**. Measurements were made in duplicate and means of the measurements are given in **Figure 2.4, 2.5 and 2.6**.

As total calcium and ionised calcium were being measured in samples using widely different methodologies it was important to validate these two techniques. A stock 5 mM calcium chloride standard was made up in 0.05 M Trizma buffer, pH 7.4, and diluted with deionised water to give standards 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4.0, 4.5 and 5mM. A further set of standards was made up over the same range that contained 3% (w/v) bovine serum albumin. The aim of using buffer solution containing albumin was to investigate the effect of a physiological protein concentration on measurements of total calcium and to check that the automatic analyser would correctly measure reduced ionised calcium levels as the albumin bound up the calcium. The two sets of standards were then measured using both techniques as described above.

Results. The validation results showed that bovine serum albumin (BSA) had no detrimental effect on the measurement of total calcium using the Wako kit on the plate reader (see **Figure 2.4**).

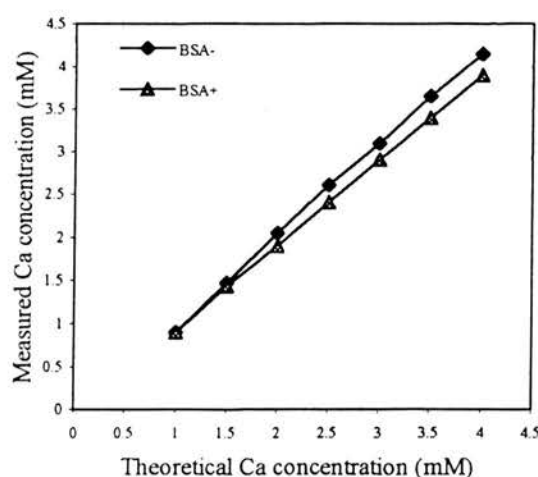


Figure 2.4. The measurement of total calcium in buffered solutions of CaCl_2 with or without 3% (w/v) added BSA.

The addition of BSA slightly improved ($P>0.05$) the accuracy of the results closer to the theoretical concentrations as the method tended to slightly overestimate calcium chloride when in solution alone. The ionised calcium meter was very accurate at estimating over the physiologically important range *i.e.* 0.5-2mM, but then

overestimated at higher concentrations (Utomo and Mitchell, unpublished data; Rennie, 1994).

As expected, BSA caused a large (about 50%) reduction in ionised calcium content of samples. The percentage of ionised calcium dropped as the total calcium fell from 67% at 4 mM to 46% at 0.5 mM (Figure 2.5).

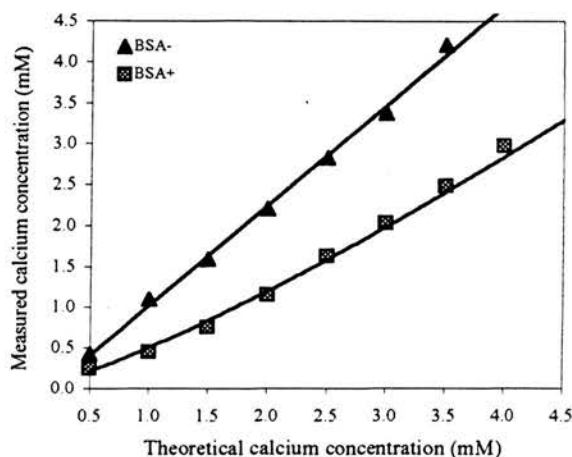


Figure 2.5. The measurement of ionised calcium in buffered solutions of CaCl_2 , with or without 3% (w/v) added BSA.

It was therefore concluded that the Wako method was completely satisfactory for measuring total calcium in plasma over a wide range. The ionised calcium meter was also completely satisfactory for measuring ionised calcium in blood over a much narrower range (Utomo and Mitchell, unpublished; Rennie, 1994). The overall correlation between the two methods over the range 0.5-5 mM was satisfactory ($r^2 = 0.99$; see Figure 2.6) assuming a linear response, and the slope of the regression line was 0.44.

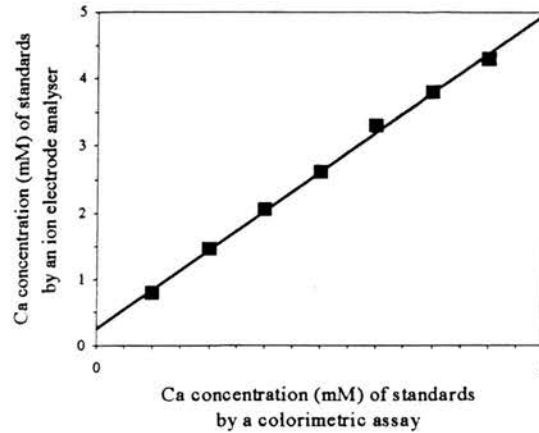


Figure 2.6. Correlation over a range of concentrations between determination of calcium obtained by a colorimetric method or a calcium-specific ion electrode.

2.4.7. Determination of plasma alkaline phosphatase activity

Plasma alkaline phosphatase (ALP) is a measure of osteoblastic activity in the chicken as in the other species (Bell, 1971). In a study of the response of plasma ALP to calcium intake in the fowl Hurwitz and Griminger (1961) found that there is a positive relationship between plasma ALP activity and calcium for the eggshell formation and egg production in the hen. It is also well known that a low dietary calcium may lead to increasing calcium bone turnover and loss (Reichmann and Connor, 1977; Taylor and Dacke, 1984; Etches, 1987).

Alkaline phosphatase is an iso-enzymes which are present in plasma derived from bone, small intestine and liver. These iso-enzymes can be separated by electrophoresis. Rosalki and Foo (1984) suggested that wheat germ (*Triticum vulgaris*) lectin precipitates bone ALP from human plasma. It, therefore, can be used to qualify both bone iso-enzyme and those from other tissues. In addition, 1-p-bromotetrasomisoile (BTM) inhibits liver and bone enzymes, and therefore, can be used to determine ALP of intestinal origin (Kuwana and Rosalki, 1990).

In the determination of plasma ALP activity all plasma samples were diluted 10-fold with distilled water (v/v) to adjust to the standard curve and the results were corrected appropriately (multiplied by 10). The activity of plasma ALP was measured

by means of a commercial kit (ALP, Wako Chemicals GmbH). The stock standard (5mM) was made by adding 10.43 mg of p-nitrophenol to one bottle of buffer (1M ethanolamine, 1 mM magnesium chloride, pH 9.8). 0.2 ml of the stock standard were added to 1.8 ml of buffer (10-fold dilution) to give the top standard (500mM). The standards were prepared by a halving dilution of 500 mM with distilled water (v/v) to give 250, 125, 62.5, 31.25, 15.625 and 7.8125 mM. But only the last four standards were used in the assay. A 260 μ l standards and blanks while 10 μ l diluted plasma were pipetted in duplicate to wells. The colour reagent was made up by adding one bottle of p-nitrophenyl phosphate substrate to one bottle of buffer. A 250 ml colour reagent was quickly added to wells containing the sample only and incubated in a water bath at 37°C for 30 min. The tubes were then centrifuged at 4000 *g* for 10 min. A precipitate was clearly visible. The absorbance was read on a plate reader for 10 min at a wavelength of 405 nm. The colour reagent was stable for up to 1 h. The results were calculated by subtracting the mean blank from the mean standard at $T = 1$ min and optical density (OD) regressed on concentration (mM). Samples were calculated using mean rate of change in OD (Δ OD/min) multiplied by a correction factor of 260 (this took into account the dilution factor of $\times 10$).

2.4.8. Determination of plasma glucose

It is one of the birds' strategy to reduce their heat production by decrease food intake voluntarily in the range of 25-35% less than birds kept under thermoneutral environment (Smith, and Oliver, 1972; Francis, MacLeod and Anderson, 1991; MacLeod, *et al.*, 1982). This means that the birds reduce their energy intake during exposure to high ambient temperature. The determination of plasma glucose is aimed to evaluate the changes of energy balance.

A commercially available kit (Glucose C, Wako Chemicals GmbH) was used for the quantitative determination of glucose in plasma. This assay depends on (1) the oxidation of D-glucose to D-gluconic acid with the simultaneous generation of hydrogen peroxide and (2) the colourimetric measurement of the hydrogen peroxide formed. The oxidation of D-glucose is catalysed by glucose oxidase which is specific

for the β -anomer of D-glucose. The Glucose C assay is unaffected by other blood constituents and can be performed without deproteinisation.

Principles of test. The equilibrium between the anomers of D-glucose in aqueous solution is maintained at a ratio of α -D-glucose 36.5% and β -D-glucose 63.5%. Glucose oxidase acts on only β -D-glucose. When a test sample is allowed to react with the reagent, α -D-glucose existing in the sample is converted rapidly to the β -anomer by mutarotase and is then oxidised by glucose oxidase to produce hydrogen peroxide. In the absence of mutarotase, the reaction proceeds slowly because existing β -D-Glucose is first consumed in the reaction catalysed by glucose oxidase and at the same time α -D-glucose is gradually converted to β -D-glucose. When mutarotase is added the conversion of α -D-glucose into β -D-glucose is accelerated so that the glucose oxidase reaction time is shortened. The hydrogen peroxide produced as a result of glucose oxidase action participates in the oxidative condensation of phenol and 4-amino-antipyrine in the presence of peroxidase to produce a red quinone dye. The amount of glucose contained in the test sample is determined by measuring the absorbance of the red colour at its maximal absorption wavelength of 505 nm.

The colour reagent solution was prepared by dissolving the entire contents of the colour reagent in the full amount of buffer solution (150 ml). The colour reagent solution was stable for 3 weeks (at 2 - 10°C). A 2 μ l of standard, sample and blank were pipetted and 300 μ l was added and mixed well in each well on a micro plate (see **Table 2.6** for the summary). The mixture was then incubated at 37°C for 5 min before being read on a plate reader at 505 nm.

Table 2.6. Procedure for the assay of plasma glucose.

	Plasma	Standard	Blank
Sample	2 μ l (Plasma)	2 μ l (Standard solution)	2 μ l (Distilled H ₂ O)
Colour reagent	300 μ l	300 μ l	300 μ l

The results were calculated by employing formula as follows:

$$\frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times C_{\text{Standard (mg/dl)}} = C_{\text{Sample (mg/dl)}}$$

Where: A = Absorbance at 505 nm

C = Glucose concentration of sample (mg/dl)

2.4.9. Determination of plasma macroprotein

The concentration of plasma macroprotein was determined using a commercial kit (Macro-protein, Bio-Rad) using standards in the range 2 to 0.125 mg/ml. The colour reagent concentrate was diluted 5-fold (1 in 4), and 50-fold (1 in 49) for plasma samples with ion-free water. The protein standard (Gammaglobulin 2 mg/ml) was diluted by way of halving dilution to give the following standard concentrations according to the protocol in **Table 2.7**. A 5 µl of standards, blank and samples were pipetted into each well and 250 µl of diluted colour reagent was added thoroughly. The colour was allowed to develop for 20 min at 37°C before being read on a plate reader at 505 nm. The plates were read within 1 h for optimum results.

Table 2.7. The procedure for the assay of plasma macroprotein.

Standard	Plate-well No.	Gamma-globulin STD (µl)	H ₂ O (µl)	Protein conc. (mg/ml)
Blank	A1 + B1	0	250	0
SO1	C1 + D1	250	0	2.0
SO2	E1 + F1	250	250 = (a)	1.0
SO3	G1 + H1	250(a)	250 = (b)	0.5
SO4	A2 + B2	250(b)	250 = (c)	0.25
SO5	C2 + D2	250(c)	250 = (d)	0.125

2.5. STUDIES *IN VIVO*: MEASUREMENT OF VITELLOGENIN UPTAKE BY OOCYTES

In the establishment of a new technique in this study all protocols for the measurement of vitellogenin uptake in the ovary were set up from the very early stage and modified at any step for the validations required. The study used column chromatography on Agarose gel and radioactive zinc (^{65}Zn) for labelling the vitellogenin. Vitellogenin binds zinc strongly as reported by Mitchell and Carlisle (1991). Zinc sticks to vitellogenin and therefore radioactive zinc (^{65}Zn) can be applied to label the vitellogenin in this study.

2.5.1. Preparation of radiolabelled ^{65}Zn -vitellogenin

2.5.1.1. Purification of vitellogenin

For the first instance an *in vivo* technique was employed as a trial to label the vitellogenin with ^{65}Zn . In this trial in order to yield a stock of ^{65}Zn labelled-vitellogenin 3 laying hens were injected intravenously with up to 1.85MBq of radioactive ^{65}Zn (as ZnCl_2). After allowing 2 hours of equilibration of *in vivo* vitellogenin zinc pool the birds were killed and blood collected by exsanguination. Radiolabelled vitellogenin was isolated and concentrated from plasma by means of centrifugal ultra-filtration. It was, however, found that this *in vivo* method was not satisfactory since the concentration of radio labelled ^{65}Zn vitellogenin obtained was very low. It would require a large volume of labelled vitellogenin to be injected to the hens. The technique was insufficient to achieve the objective and it was discontinued.

Instead, an *in vitro* labelling method was developed. In this trial five laying hen plasmas (± 12 ml) were labelled *in vitro* by adding 1.85MBq of radioactive ^{65}Zn (as ZnCl_2). Radiolabelled vitellogenin was then concentrated from plasma by centrifugal ultra-filtration and a vitellogenin enriched (purified) fraction was prepared by gel chromatography.

2.5.1.2. Procedure of chromatography by gel filtration

A semi-purified and labelled vitellogenin was isolated and fractionated by gel filtration. The chromatography column buffer contents 154 mM NaCl (0.9% sodium chloride), 20mM TRIS, 1mM EDTA and 50 µg/ml PMSF in two litres of distilled water adjusted to pH 7.4 by adding 20mM HCl.

A gel with molecular weight exclusion of 5 million (Bio-Gel A-5 M, Bio-Rad; Agarose beads for gel filtration) was used (since MW of vitellogenin previously reported is 4800 000). It was an Agarose gel in the form of spherical beads, specially prepared for gel filtration in aqueous media, fully hydrated in 10mM EDTA with 0.04% sodium azide as a preservative. The gel was mixed until no settled solution remained and some freshly made chromatography column buffer was added and shaken. The solution was poured into the column with the bottom tap open to collect the buffer from the tap with a beaker. The gel was allowed to settle but not dry out and more was added until the column was almost full. Some clean fresh buffer was then added to about 2-3 mm as a layer on top (2-3 Pasteur pipette's full). The bottom tap was closed after the drips had stopped. The pump was then connected to the column, the tap opened and the effluent buffer was collected (see **Figure 2.7**). A steady flow rate was established. Finally, a fraction collector was connected up and the labelled vitellogenin was loaded onto the gel.

2.5.1.3. Principle of gel (exclusion) chromatography

Chromatography by gel filtration technique is based on the size of molecules. The immobile phase was constituted by an Agarose gel, in the form of spherical beads, specially prepared for gel filtration in aqueous media. The exclusion limit was 5,000,000 molecular weight (MW); because the operating range was 10,000 to 5,000,000 MW, therefore the column could separate any solution containing molecules within that range. The mobile phase was a buffer containing: 154mM NaCl, 20 mM Tris and PMSF (50 mg/ml) which was adjusted to pH 7.4 with 20mM HCl. A pump was connected to the column which then pumped the buffer through the gel. and

effluent buffer coming out was collected in tubes in a fraction collector. The column dimensions were 45 cm high and 3 cm in width as illustrated on **Figure 2.7**. The standard calibration solution contained 4 proteins (of which one was coloured) and two coloured markers (see **Table 2.8**). The coloured markers were helpful in detecting the volume of elution for the standards and to check the column for good running, no air bubbles and good seating. Eluents were assayed for protein (vitellogenin and VLDL) to determine the elution profiles which were then plotted.

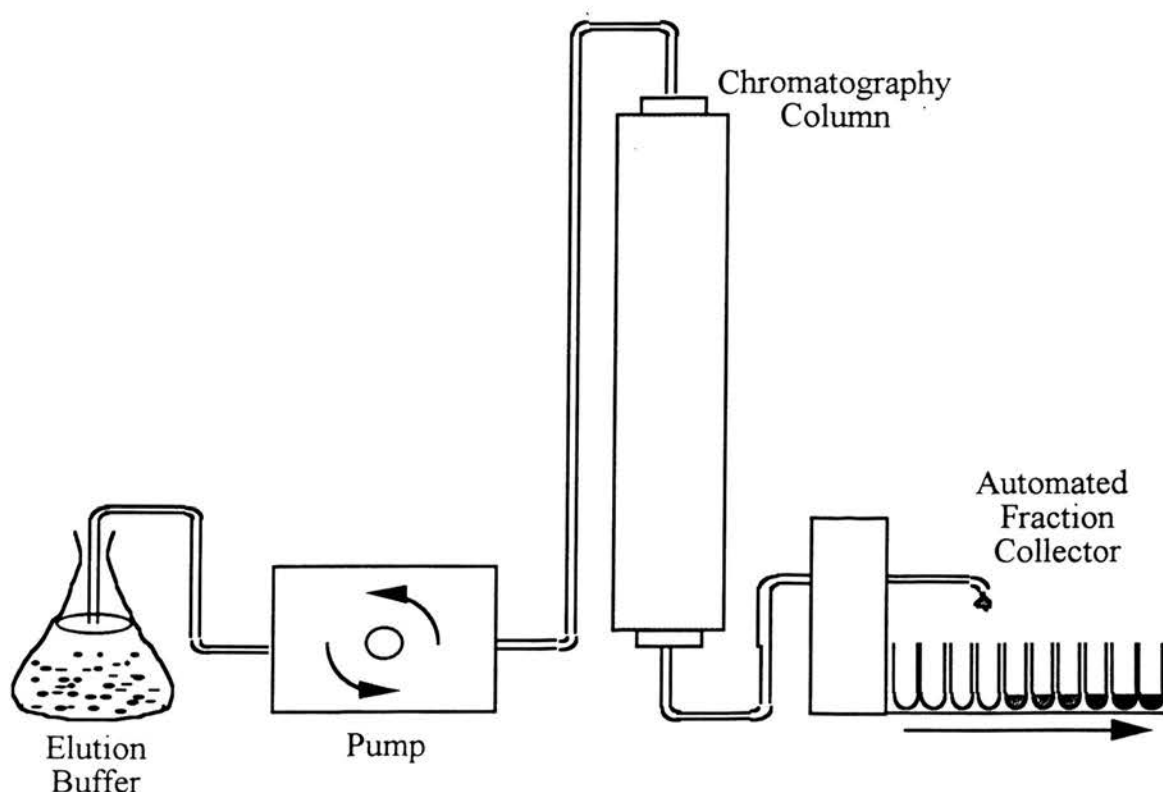


Figure 2.7. Illustration of the chromatography column

2.5.1.4. Calibrating the gel and elution profile of the fractions

Before the column was ready to use in the chromatography it was necessary to calibrate the column. The column was run overnight using a Bio-Rad gel calibration standards to determine the log MW-elution volume profiles approximate V_0 and

elution volume required for low MW plasma components (<2.0kDa). The elution was determined by visual observation of the appearance of the colour (see **Table 2.9**). Labelled plasma was then loaded into the column and run for the gel filtration chromatography. All the fractions were collected and protein, zinc and triglyceride assays were done on all fractions to determine the elution profiles. The results of the elution profiles were then plotted.

Table 2.8. Composition of the chromatography standard solution

Markers	Quantity (for 2 l buffer)	Molecular weight (Da)	Colours
Dextran blue	1 mg	2.00×10^6	Blue
Thyroglobulin	5 mg	0.67×10^6	-
Bovine γ -globulin	5 mg	0.16×10^6	-
Chicken ovalbumin	5 mg	44×10^3	-
Equine myoglobin	2.5 mg	17×10^3	Brown
Vitamin B ₁₂	0.5 mg	1350	Purple

2.5.2. Injection of radiolabelled ^{65}Zn -vitellogenin into birds

In order to study the distribution of the protein and to estimate the uptake rate by individual oocytes, laying hens were injected intravenously through the brachial vein with semi-purified ^{65}Zn -labelled vitellogenin obtained from other hens. Following a period to allow equilibration of the isotope with the *in vivo* vitellogenin zinc pool; blood samples were taken at 2, 15, 30, 45, 60 and 90 min after injection and collected into heparinised tubes. Previous *in vivo* trials showed that the equilibration of the isotope was achieved within 2 min and the isotope completely disappeared from the circulating blood within 90 min after injection. The birds were then killed and the oocytes were collected, weighed and measured for radioactivity using a γ -counter (^{65}Zn was a high energy γ -emitter and was easily detected and quantified in tissue and fluid samples).

2.5.3. Detecting and quantifying in tissue and fluid samples

The distribution of the labelled ligand was determined in a number of tissues and plasma and within the oocyte. The whole blood, plasma, liver and oocytes obtained were then counted on a γ -counter (1277 GammaMaster, LKB-Pharmacia). Each tissue was weighed, homogenised and counted with the appropriate counting geometry. The oocytes were also measured for their mass, density and surface area in order to calculate the uptake per gram tissue and per cm² of oocyte surface and per gram tissue per kg body weight. The rates of uptake and tissue distributions and elimination rate of ⁶⁵Zn -vitellogenin were also determined.

2.6. HORMONAL DETERMINATIONS

Plasma hormone concentrations were estimated by radioimmunoassay (RIA) and calculated by using a computer program AssayZapTM (AssayZap Universal Assay Calculator, Biosoft, Cambridge UK). With the exception of the totals all supernatant fractions were aspirated to waste and the totals and pellets counted for 60 seconds on a gamma-counter (1277 GammaMaster, LKB-Pharmacia). The data obtained were transferred into AssayZapTM. The computer program was designed to linearise the standard curve using a logit-log transformation. All the samples of a particular study were assayed at the same time.

2.6.1. Radioimmunoassay of 17 β -oestradiol

Laying hens were injected subcutaneously with 17 β -oestradiol (Sigma Chemical Co., Poole, UK) dissolved in methanol and diluted with olive oil at the dose rate of 5 mg/kg body weight daily for seven days prior to blood sampling. The control birds received the olive oil only. The commercial kit (DSL ActiveTM

Oestradiol, Sigma Chemical Co., Poole, UK) was used for the quantitative measurement of oestradiol in plasma.

2.6.1.1. Extraction for modification of 17β -oestradiol assay

Plasma 17β -oestradiol was measured by means of the coated tube technique (Active™ Estradiol, Biogenesis Ltd., Poole, UK) with some modifications (Mitchell and Utomo, unpublished data). All test tubes and glassware were washed in ion-free water. The basic principle of radioimmunoassay was the competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. The amount of [I-125] labelled analyte bound to the antibody was inversely proportional to the concentration of the analyte present. The separation of free and bound antigen was easily and rapidly achieved by decanting or aspirating the antibody coated tubes.

2.6.1.2. Preparation of the standards

Stock solutions of [0, 20, 50, 250, 750, 1500, 3000 pg/m]-oestradiol- 17β standards were prepared in duplicate in coated tubes except tubes 3 and 4 which were LP4 tubes for the Non-Specific bindings (NSBs). A standard curve was set up for each radio-immunoassay as detailed in **Table 2.9**.

2.6.1.3. Radioimmunoassay procedure

All liquid reagents were allowed to reach room temperature and vortex-mixed thoroughly by gentle inversion before assay. Standards, controls and unknowns were assayed in duplicate. Anti-oestradiol coated tubes were labelled and arranged in duplicate according to the protocol shown in **Table 2.9**. A 100 μ l of the oestradiol standards, controls and samples were pipetted into the bottom of the appropriate

tubes. To uncoated plastic NSB tubes were added 100 µl of the 0 pg/ml oestradiol standard. 500 µl of oestradiol [I-125] reagent were added to each tube and vortexed. All tubes were then covered and incubated in a waterbath at $37 \pm 2^\circ\text{C}$ for 2 h. All tubes, except total-count tubes, were aspirated and the tubes were stood sharply on absorbent material to facilitate complete drainage. They were drained on absorbent material for 5-10 min before blotting the tubes to remove any droplets adhering to the rim; then returned to the upright position. All tubes were counted in a gamma counter for one minute.

The result was determined by calculating the average counts per min (CPM) for each standard, control and unknown. The average CPM of the NSB tubes was subtracted from all counts to obtain corrected counts. The % bound or %B/Bo for each standard, control and unknown were calculated as follows:

$$\% \text{ B/T} = \frac{\text{Sample Counts} - \text{NSB Counts}}{\text{Average Total Counts}} \times 100$$

$$\% \text{ B/Bo} = \frac{\text{Sample Counts} - \text{NSB Counts}}{\text{Average Counts of 0 pg/ml Standard} - \text{NSB Counts}} \times 100$$

A curve of radioactivity counts per minute (CPM), % bound or % B/Bo for the oestradiol standards was plotted against the oestradiol concentration to determine the oestradiol concentration using AssayZapTM. However, since a laying hen plasma has a very high lipid content, which influences the process of antigen-antibody bindings, it is difficult to obtain the results although the standard results give a good curve, but not for the samples. A lot of assays were carried out with some modifications were in vain, but the problem was not elucidated yet.

Although several trials and attempts to modify the commercial kit available have been carried out to determine the oestradiol concentration in the plasma laying hens, it was still unable to obtain a satisfied result. Attempts was still being made in modification and extraction of abundant fatty acids from plasma of laying hens.

Table 2.9. Summary of 17β -oestradiol radioimmunoassay protocol.

Tube No.	Tube contents	Standards/ Controls/ Unknowns (μ l)	Oestradiol [I-125] reagents (μ l)
1,2	Total counts	Nil	500
3,4	NSB	100*	500
	Oestradiol Standards (pg/ml)		
5,6	0	100	500
7,8	20	100	500
9,10	50	100	500
11,12	250	100	500
13,14	750	100	500
15,16	1500	100	500
17,18	3000	100	500
	Oestradiol Controls		
19,20	Level I	100	500
21,22	Level II	100	500
NSB = Non-Specific Binding * = 0 pg/ml oestradiol standard (were not antibody coated tubes)			

2.6.2. Radioimmunoassay of triiodothyronine and thyroxine

Both the triiodothyronine (T3) and thyroxine (T4) assays were measured by means of commercial coated tube kits (Pantex thyroid hormone assays kit [CT] - Biogenesis, UK). The assays were developed and optimised for clinical use in humans and this was reflected in the concentration ranges employed in the standard curves. The plasma concentrations of T3 and T4 encountered in birds were usually in the ranges of 0.5-5.0 and 5-25 ng/ml respectively. It was therefore necessary to make

some slight modifications to the assay protocols to make them suitable for use with avian plasma samples.

Triiodothyronine assay procedure. Calibrators or standards were reconstituted with de-ionised water. The kit range was from 0 to 8 ng/ml and the lowest was 0.25 ng/ml. The kit standards were used and kit assay protocol followed exactly as per specification. Standards or samples were dispensed into pre-coated tubes in a volume of 100 μ l and mixed with 1.0 μ l of tracer (see also **Table 2.10.** for the summary). The tubes were then incubated at 37°C for 2 h and the liquid phase aspirated. The T3 plasma concentration was determined by counting. A curve of radioactivity counts per minute (CPM), % bound or %B/Bo for the triiodothyronine standards was plotted against the triiodothyronine concentration to determine the triiodothyronine concentration using AssayZap™.

Thyroxine assay procedure. Calibrators or standards were prediluted but the range was inappropriate for use with avian plasma. The three top standards (320, 160 & 80 ng/ml) were removed from the kit and labelled with kit number, dated, frozen and stored at -20°C. The standards at 40, 20, 10 and 0 ng/ml were used as presented in the kit. However, 250 μ l aliquots of the lowest standard (10ng/ml) were transferred to an Eppendorff microtube and diluted 2-fold with the zero standard (add 250 μ l) to produce a bottom standard of 5ng/ml with the correct protein concentration. The assay was performed as per kit protocol but 50 μ l (not 25 μ l) of standards or samples were added to the pre-coated tubes. They were mixed with 1.0 ml tracer and then incubated at 37°C for 1 h (see also **Table 2.11** for the summary). The liquid phase was determined by counting and T4 plasma concentration was determined as in the T3 procedure.

Table 2.10. Summary of triiodothyronine (T3) radioimmunoassay protocol.

	(A) tubes	(1) cal or sample	(2) tracer
TC cal (ng/dl)	plain	0 μ L	1000 μ L
0	1,2	100	1000
25	3,4	100	1000
50	5,6	100	1000
100	7,8	100	1000
200	9,10	100	1000
400	11,12	100	1000
800	13,14	100	1000
control 1	15,16	100	1000
control 2	17,18	100	1000
sample 1	19,20	100	1000

Table 2.11. Summary of thyroxine (T4) radioimmunoassay protocol.

	(A) tubes	(1) cal or sample	(2) tracer
TC cal (ng/dl)	plain	0 μ L	1000 μ L
0	1,2	25	1000
1	3,4	25	1000
2	5,6	25	1000
4	7,8	25	1000
8	9,10	25	1000
16	11,12	25	1000
32	13,14	25	1000
control 1	15,16	25	1000
control 2	17,18	25	1000
sample 1	19,20	25	1000

Assay principles of T3 and T4. The set of calibrators and fixed amounts of radiolabelled thyroxine tracers (triiodothyronine or thyroxine) competed for fixed amounts of binding sites on anti-thyroxine antibodies coated on tubes. Ligand (triiodothyronine or thyroxine) from sera or calibrators reduced tracer binding on tubes. The basis of radioimmunoassay was the quantitative relation between ligand concentration and proportion of tracer bound to anti-serum. Tracer bound to the walls was counted after supernatant removal. Calibrator binding values were plotted vs. concentrations. The best line was drawn between points. Control or sample binding values interpolated on the line gave T3 or T4 concentrations. The results were calculated using AssayZapTM as the calculation of oestradiol radioimmunoassay (see Section 2.6.2 for details).

2.7. GENERAL STATISTICAL ANALYSIS

In the most experiments of this study there were two variables for the chamber's temperature (thermoneutral and heat stress) and some treatments, *i.e.* diets, feeding schemes and oestradiol challenge. Separate analysis was employed for adaptation period to evaluate whether there was a difference between groups and/or between chambers before the treatments. Then using the remaining periods to fit the effects of heat stress and/or other treatments and interaction between heat stress and treatments. It is, therefore, all of the experimental designs have to be fitted to the special and appropriate arrangements being employed.

Data from experiments performed as balanced designs were analysed statistically by one-way and two-way analysis of variance considering treatment groups within each temperature. Differences among means were estimated using the Student's unpaired *t*-test for unequal numbers. Statistics were calculated using Minitab (Minitab Inc., UK), Genstat (Genstat 5 Committee, UK) and a General Statistics Package (Hewlett-Packard International, USA). Unless otherwise indicated, all data were expressed as the mean \pm either standard error of the mean (s.e.m.) or standard deviation (SD) whichever was more appropriate. Determination of statistical significance of data obtained from RIA was done by Student's unpaired *t*-test (StatWorks; Cricket Software) or analysis of variance (StatView-II, SuperAnova), where $P < 0.05$ was considered significant.

CHAPTER THREE

CHANGES IN CIRCULATING LEVELS OF EGG YOLK PRECURSOR AND EGG PRODUCTION IN HEAT STRESSED LAYING HENS

3.1. EFFECTS OF CHRONIC HEAT STRESS ON EGG YOLK PRECURSORS AND EGG PRODUCTION

3.1.1. Introduction

Yolk is the most important part of an egg and the quantity of yolk produced is a major factor in the determination of the ultimate size of the egg (Redshaw and Follet, 1972; Griffin *et al.*, 1984). It is also well known that thermal stress causes decreased egg production. Many studies in this area are often related to decreased egg production with down-grading and egg quality determinations with little attention on the effects of thermal stress upon egg yolk precursors and their deposition in the oocyte. It, therefore, is important to evaluate the effects of heat stress on yolk and egg production and relate the changes to the yolk precursor levels in the blood circulation. The objectives of the first study in this **Chapter Three** were therefore to determine the effects of high ambient temperature (32°C with 35% RH) on laying performance in comparison with those kept at a thermoneutral temperature (21°C with 35% RH); and to investigate the changes in the plasma levels of yolk precursor.

3.1.2. Experimental procedures

Twenty-four 35-week-old ISA brown laying hens were used in this experiment that were divided randomly into two groups. They were placed in two modules with a lighting schedule 14h light : 10h dark. Chamber temperature and relative humidity were recorded every five minute throughout the experimental periods. Control diet was a standard layers' mash containing 16% CP, 11 MJ ME and 33g Ca/kg. The

experiment was carried out for eight weeks and divided into three experimental periods, *i.e.* adaptation (22°C, four weeks), heat stress (32°C, two weeks) and recovery (22°C, two weeks) periods with a constant relative humidity of 35% throughout the experimental period. The eggs laid daily were collected individually, dated and grouped. All eggs were weighed and broken to measure the yolk weight, shell weight and shell thickness. Food was given every day and food intake was measured every other day at approximately the same time throughout the experimental periods. The deep body temperatures were measured once a week within the climate chamber under the appropriate environmental conditions at the time of blood sampling to minimise the stress of handling. The blood samplings were taken weekly during the adaptation and prior exposure to the heat stress treatment, while during heat stress there were two times blood samplings and once during the recovery period (10 days post heat stress). After the blood sampling on week 5 (**Figure 3.1**) heat stress treatment was imposed and released after the blood sampling on week 7.

The circulating concentration of vitellogenin was measured as plasma zinc (Zn) and very low density lipoprotein (VLDL) as plasma triglyceride by the methods of Mitchell and Carlisle (1991). Whilst plasma calcium (Calcium) concentration was measured by the *in vitro*-OCPC method for the quantitative determination of total calcium. The concentration of ionised calcium (Ca^{2+}) in whole blood was measured using a Ciba-Corning 634 Ca^{2+} /pH analyser. Data were analysed by ANOVA (Steel and Torrie, 1980). Total egg mass was expressed as the total weight of eggs produced by each bird as well as by group within a day, week or through the total experimental periods. Yolk mass per egg and total yolk mass produced by each bird were also determined to characterise the long term effects of heat stress.

3.1.3. Results

The effects of high ambient temperature upon rectal temperature, food intake, egg production and changes in the circulating levels of egg yolk precursors (vitellogenin and VLDL), calcium and blood pH in laying hens are summarised in **Figures 3.1 - 3.7** and **Tables 3.1 - 3.5**.

All plasma metabolite concentrations were initially presented (in line graphs) based on their weekly means throughout the eight week experimental periods. In the later comparison the means were then plotted in a three experimental period (pre-, during- and post-heat stress) bar graphs.

3.1.3.1. Changes in deep body temperature and food intake

Exposure laying hens to a chronic high ambient temperature obviously caused heat stress conditions in the birds. It was clearly observed during heat stress that the birds showed compensatory behavioural responses such as panting (it will be discussed later that panting caused in a respiratory alkalosis in this **Chapter**) and raising the wings to dissipate excess heat. A significant increase in deep body temperature was found immediately (see **Table 3.1**) on the days following the heat stress treatment. It is possibly a result of insufficient heat dissipation during heat stress which was beyond the birds' thermoregulatory capability.

Table 3.1. Changes in means of rectal temperature ($^{\circ}\text{C} \pm \text{s.e.m.}$)

Group	Pre-Heat	Heat stress	Post-Heat Stress
Treatment	$41.4^{\text{a}} \pm 0.05$	$42.2^{\text{b**}} \pm 0.09$	$41.7^{\text{a}} \pm 0.06$
Control	$41.3^{\text{a}} \pm 0.02$	$41.4^{\text{a}} \pm 0.06$	$41.6^{\text{a}} \pm 0.03$

Means with different superscripts differ significantly ($P < 0.05$) ** $P < 0.01$

It is known that in order to reduce the metabolic heat production in heat stressed birds, they tend to reduce their food intake (Mitchell and MacLeod, 1984) and increase their water consumption to assist the process of evaporative cooling (Smith and Oliver, 1971). Similar results were also observed in the present study. Heat stressed birds significantly reduced their food intake. The reduction represented a 25% decrease compared to the pre-heat stress value (**Table 3.2**).

Table 3.2. The effect of heat stress on voluntary food intake (g/d \pm s.e.m.).

Group	Pre-Heat	Heat stress	Post-Heat Stress
Treatment	159.5 ^a \pm 5.98	120.7 ^{b**} \pm 9.85	143.2 ^a \pm 8.96
Control	163.1 ^a \pm 8.93	166.4 ^a \pm 4.79	152.7 ^a \pm 6.24

Means with different superscripts differ significantly ($P < 0.05$) ** $P < 0.01$

3.1.3.2. Egg production

All eggs from both groups were collected and weighed on a daily basis and presented collectively as total weekly weight and egg production. The egg production data are also presented in terms of mean egg weight per day, mean yolk weight and total egg mass for the whole experimental period as summarised in **Table 3.3**.

Heat stress reduced egg production in both egg number and total egg mass produced. These effects included having thinner egg shell (decreased mean and percentage of shell mass per egg) and reduced yolk weight as well as egg weight in heat stressed birds ($P < 0.05$). Total weekly egg production in heat stressed group was consistently lower ($P < 0.05$) than control group. The decrease represented a significant reduction of 29% in egg production ($P < 0.01$) in heat stressed group.

Compared to control group total egg mass production was significantly lower ($P < 0.01$) in heat stressed group. This decrease represented a 35% reduction in total weekly egg mass in addition to the decreased mean egg weight (8%, $P < 0.05$) in heat stressed group. Total yolk mass produced in heat stressed group was also significantly lower ($P < 0.01$) than control group, since there was a decrease in the mean of yolk weight per bird (7%, $P < 0.05$). The observed differences were due to the difference in the number of eggs produced in the both groups.

Table 3.3. Effect of heat stress on egg production in laying hens (weekly; $n=12$).

	Pre-Heat Stress		Heat Stress		Post-Heat Stress		Pooled s.e.m.
	HS	Ctrl ^{*)}	HS	Ctrl	HS	Ctrl	
\bar{x} egg weight (g)	62.99 ^a	61.84 ^a	57.89 ^b	61.28 ^a	61.59 ^a	60.56 ^a	1.73
Σ egg number (n)	75 ^a	79 ^a	53 ^b	80 ^a	62 ^b	77 ^a	10.97
Σ egg mass (g)	4724 ^a	4885 ^a	3068 ^b	4902 ^a	3819 ^{ab}	4663 ^a	742.2
\bar{x} yolk mass per egg (g)	17.40 ^b	16.93 ^b	16.21 ^a	17.19 ^b	16.88 ^{ab}	17.34 ^b	0.44
Σ yolk mass (g)	1305 ^b	1337 ^b	859 ^a	1375 ^b	1047 ^c	1335 ^b	208.7
\bar{x} shell mass per egg (g)	6.24 ^a	6.21 ^a	5.75 ^b	6.18 ^a	6.13 ^a	6.09 ^a	0.18
Σ shell mass (g)	468 ^a	491 ^a	305 ^{b*}	494 ^a	380 ^b	469 ^a	75.9

Means in each row followed by different superscripts differ significantly ($P<0.05$).

^{*)} Ctrl is the control group which is exposed to thermoneutral (22°C) throughout the experimental periods and HS group is treated with heat stress (32°C).

3.1.3.3. Plasma egg yolk precursor concentrations

In this study the circulating levels of egg yolk precursor were mainly determined by the concentrations of its two major components *i.e.* vitellogenin and very low density lipoprotein (VLDL). Circulating vitellogenin and VLDL concentrations are represented by zinc (Zn) and triglyceride concentrations in the plasma, respectively, as described in the method established by Mitchell and Carlisle (1991). The concentrations were calculated using the linear regressions obtained from the standards given (see also **Section 2.4.1 in Chapter Two**).

In the early phase of the experiment (pre-heat stress period) there was no difference in both plasma vitellogenin and VLDL concentrations between the two experimental groups. However, chronic exposure to high ambient temperature (32°C; 35% RH) for two weeks significantly decreased both plasma vitellogenin and VLDL concentrations (**Figure 3.1**). Therefore, highly significant differences were found between groups during the heat stress period ($P<0.001$).

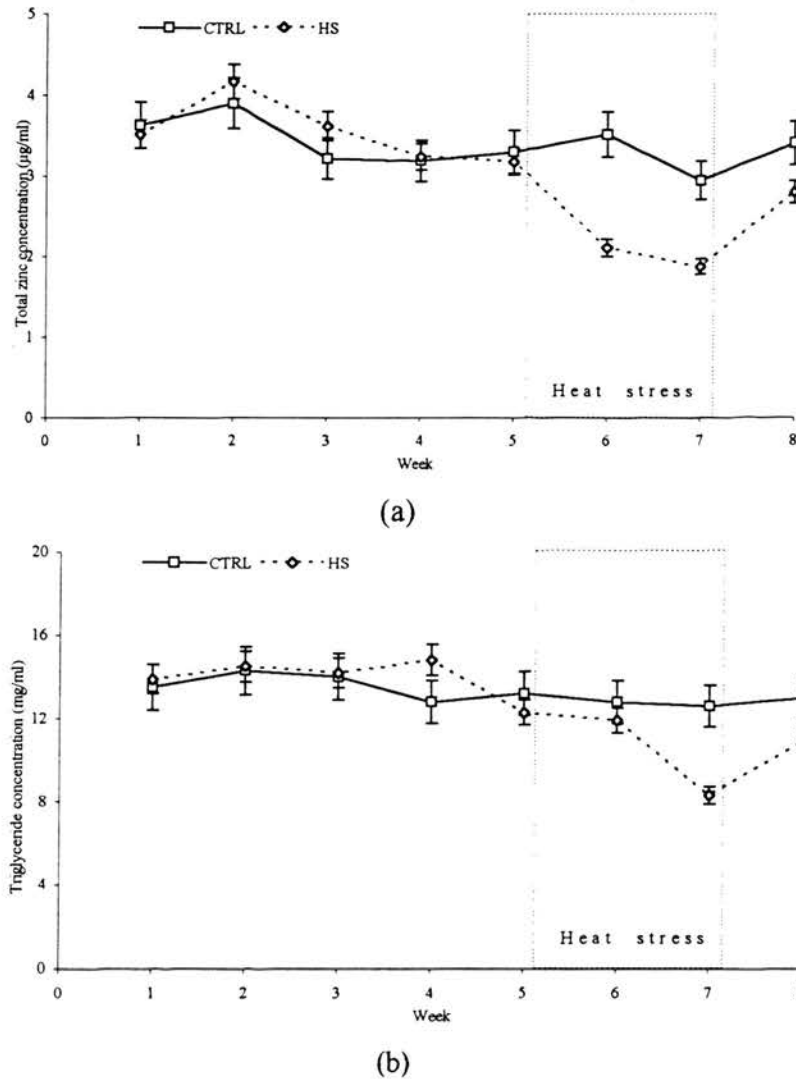


Figure 3.1. The effects of heat stress for two weeks (32°C with 35% RH; week 5-7) following a four week adaptation upon (a) total zinc (vitellogenin) and (b) triglyceride (VLDL) concentrations in the plasma of laying hens (values represent mean \pm s.e.m.; $n=12$). After the blood sampling on week 5 the heat stress was imposed which was released after the blood sampling on week 7.

The pattern of changes in the circulating level of VLDL in heat stressed birds however was not similar with those in vitellogenin. Vitellogenin concentration fell immediately after the exposure of the birds to heat stress and kept decreasing during that period. Whilst VLDL concentration slightly decreased in the first week of heat stress, the reduction was less severe compared to those in vitellogenin, but dramatically dropped during the second week of exposure. The data showed non similar numerical decreases. The effects of heat stress on egg yolk precursor

concentrations were more clearly observed when the results were plotted into three experimental periods as shown in **Figure 3.2**. The reduction of plasma vitellogenin and VLDL concentrations during the heat stress period represented 34% and 29% reductions respectively. The cumulative values showed that the reduction in VLDL was less severe compared with those in vitellogenin. The pattern of the changes in egg yolk precursors were similar with those changes in the body temperature and food intake during heat stress period, but not in the recovery period. During the recovery period there were still differences in egg yolk precursor concentrations whereas the body temperature and food intake were not.

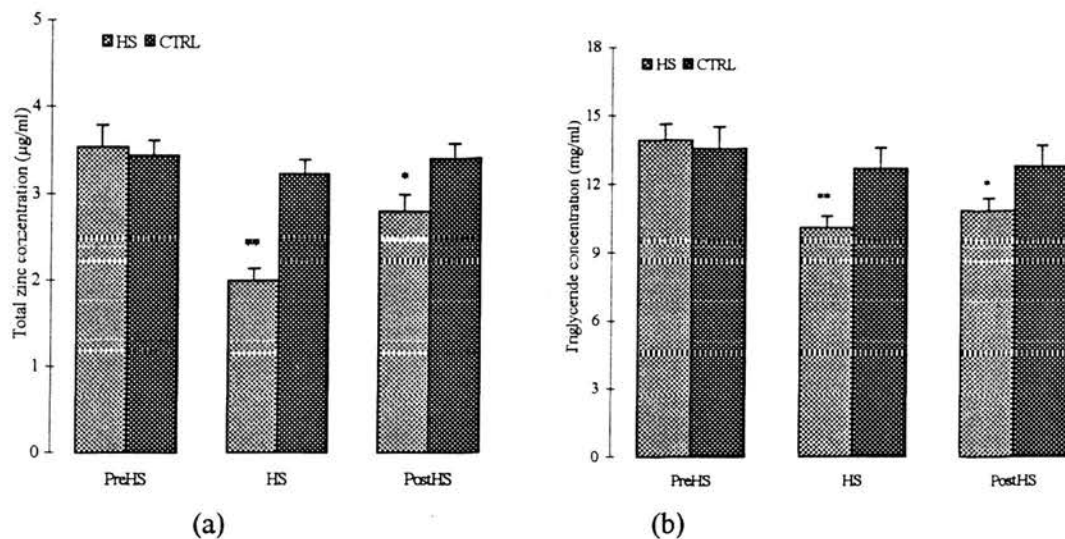
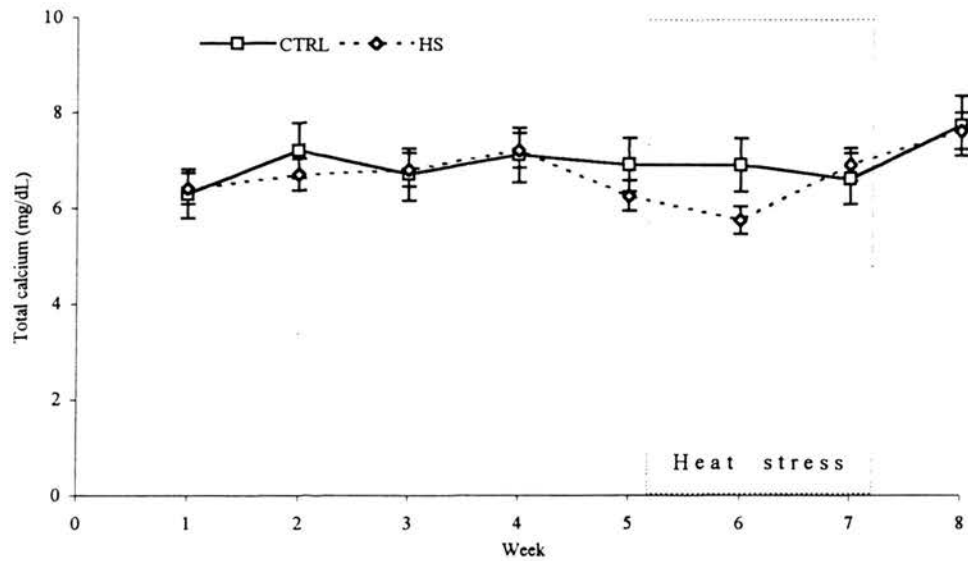


Figure 3.2. The effects of heat stress (32°C with 35% RH for two weeks) following a four week adaptation upon (a) total zinc and (b) triglyceride concentrations in the plasma of laying hens. Values represent the means within each period (\pm s.e.m; $n=12$).

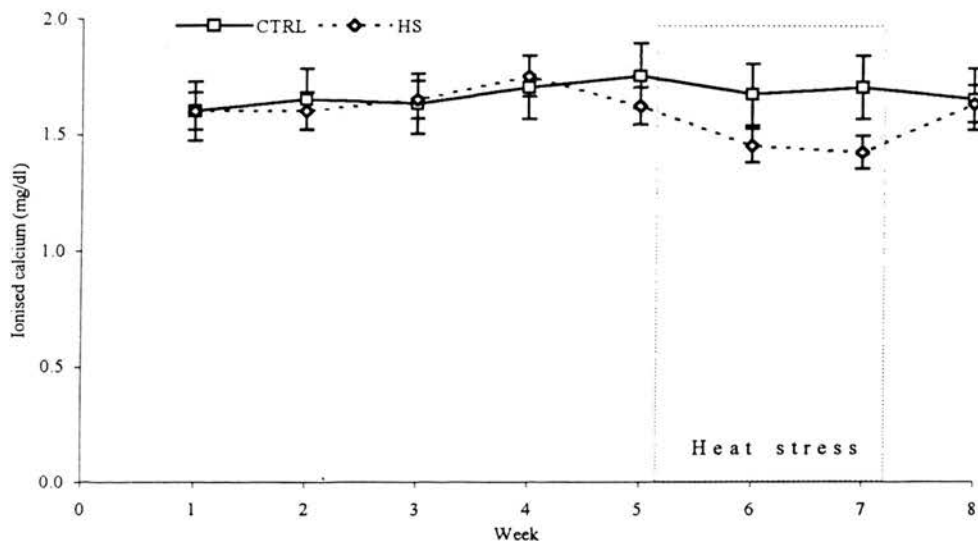
3.1.3.4. Plasma total and ionised calcium concentrations

The results of this study also shows that heat stress decreases the concentrations of ionised calcium ($P<0.01$) but not total calcium in the blood stream of laying hens (**Figure 3.3**). The changes in ionised calcium concentration concomitant with the increase in the blood pH (to be discussed later on the next section) *i.e.* a respiratory alkalosis occurred, very likely due to panting which increases CO₂ losses during heat

stress. The concentration of ionised calcium was back to normal level in the recovery period. This study confirms that ionised calcium is more responsive to heat stress than total calcium. **Figure 3.4**, which represents the means of both total and ionised calcium in each period, shows their different responses to heat stress.



(a)



(b)

Figure 3.3. The effects of heat stress (32°C with 35% RH; for two weeks) following a four week adaptation upon (a) total calcium and (b) ionised calcium concentrations in the plasma of laying hens. Values represent mean \pm s.e.m. from 12 birds. Notice the difference between both graphs in the scale of Y axis. The heat stress was imposed after the blood sampling on week 5 which was released after the blood sampling on week 7.

The concentrations of ionised calcium were decreased during exposure to high ambient temperature was also reported by De-Andrade *et al.* (1977), and related to respiratory alkalosis during heat stress. The present results may indicate a decrease in availability of calcium for egg shell formation during heat stress as suggested by Odom, *et al.* (1986). In addition, the pattern of decreased in ionised calcium was similar to the pattern of decreased in egg production. Reducing blood ionised calcium may in turn limit the availability of calcium for eggshell formation (Odom, *et al.*, 1986).

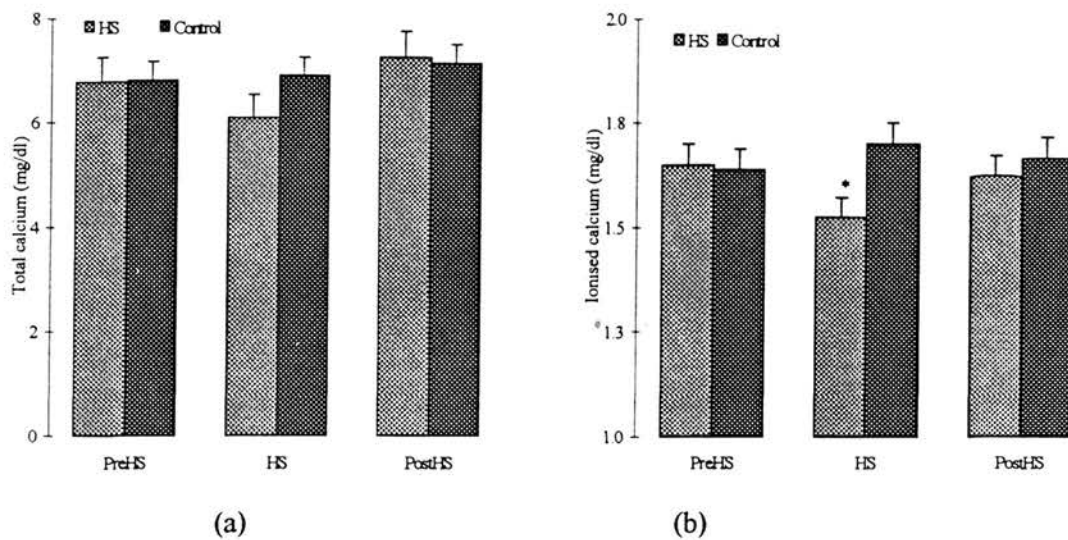


Figure 3.4. The effects of heat stress (32°C with 35% RH for two weeks) following a four week adaptation upon plotted in three experimental periods (a) total calcium and (b) ionised calcium concentrations in the plasma of laying hens. Values represent the means \pm s.e.m.; ($n=12$). Notice the difference between both graphs in the scale of Y axis.

3.1.3.5. Blood pH

Birds exposed to the high ambient temperature exhibited a heat stress and had disturbances in acid-base balance resulting in an increase in the blood pH (Figure 3.5) *i.e.* a respiratory alkalosis.

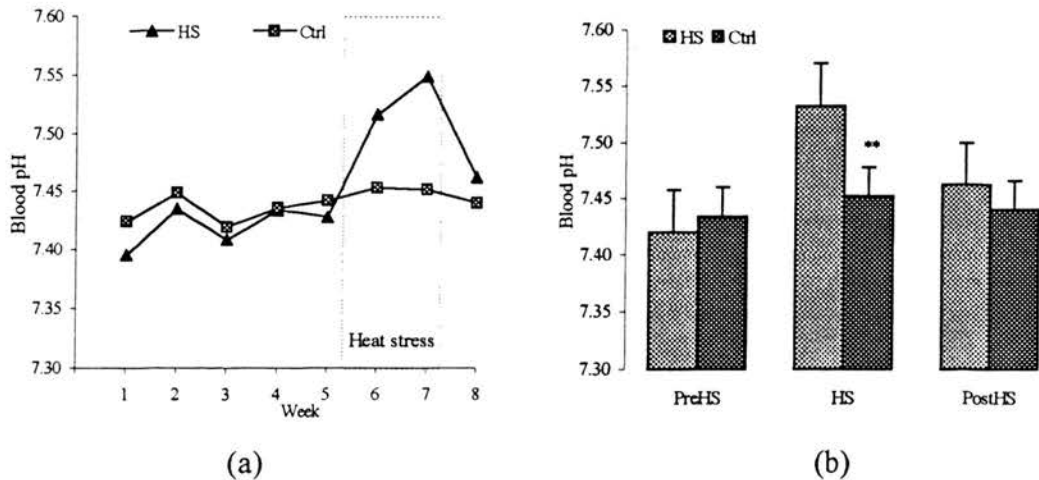


Figure 3.5. The effects of heat stress (32°C with 35% RH for two weeks) upon blood pH in the plasma of laying hens (a) following a four week adaptation . Values represent the weekly means (\pm s.e.m.; $n=12$) and (b) plotted in a three experimental periods graph. Heat stress treatment was imposed after the blood sampling on week 5 which was released after the blood sampling on week 7. Values represent the means within each period (\pm s.e.m.; $n=12$).

3.1.3.6. Plasma inorganic phosphorus concentration

Figure 3.6 shows that before chronic heat stress treatment, there was no difference in plasma inorganic phosphorus concentration between the two experimental groups. However, over the following five weeks of adaptation there was an overall significant reduction in plasma inorganic phosphorus concentration in treated birds. The circulating inorganic phosphorus in heat stressed birds was significantly lower ($P<0.01$) compared to those in control group. The reduction in treated groups was more severe in the first week than in the second week of heat stress (Figure 3.6a). The decrease represented approximately a 40% reduction in heat stressed group (Figure 3.6b).

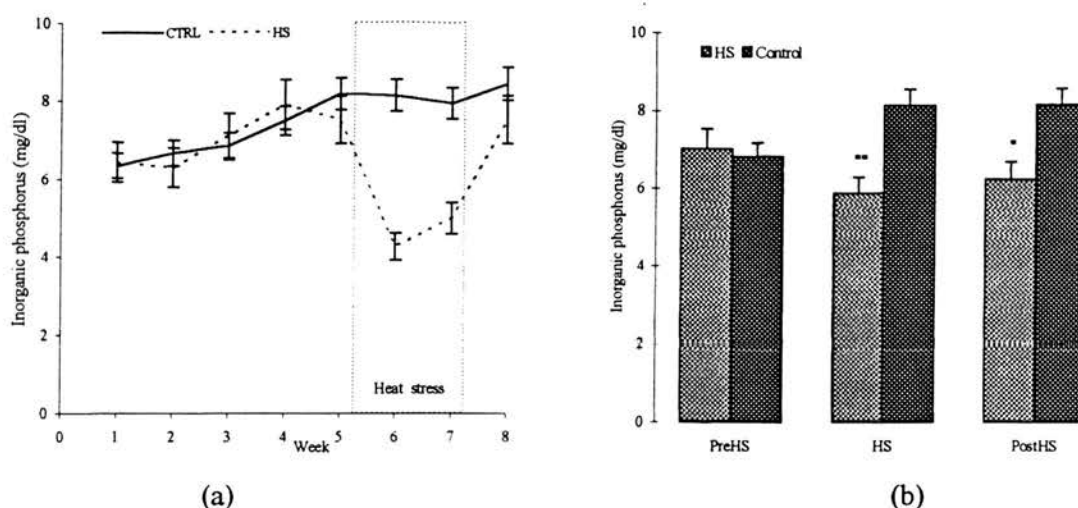


Figure 3.6. The changes in the circulating level of inorganic phosphorus in the plasma of chronically heat stressed laying hens (32°C with 35% RH for two weeks); (a) following a four week adaptation. Heat stress treatment was imposed after the blood sampling on week 5 which was released after the blood sampling on week 7. Values represent the weekly means; and (b) plotted in a three experimental period graph. Values represent the means \pm s.e.m.; $n=12$.

The observed difference in the percentage reduction of the plasma inorganic phosphorus concentration is similar to the work reported by Aitoulahsen *et al.* (1989). They concluded that changes in plasma concentration of major ions occurred during heat stress. Increased plasma sodium and chloride, decreased plasma potassium and phosphate were observed in birds exposed to high ambient temperature.

The differences in plasma inorganic phosphorus concentration between control and heat stressed groups was still found during the recovery period at the end of the experiment. **Figure 3.6** also shows that the circulating inorganic phosphorus was still significantly lower in the recovery period. However, it seems that in the recovery period heat stressed birds took a long time to get back to normal level as it was before exposure to chronic heat stress.

3.1.3.7. Plasma glucose concentration

The result of this study shows that heat stress has no effect on the plasma glucose concentration ($P>0.05$). This applied to the both the weekly values and in

each experimental period glucose concentration (**Figures 3.7a and 3.7b**). There were no significant differences between chronically heat stressed groups and those in control group.

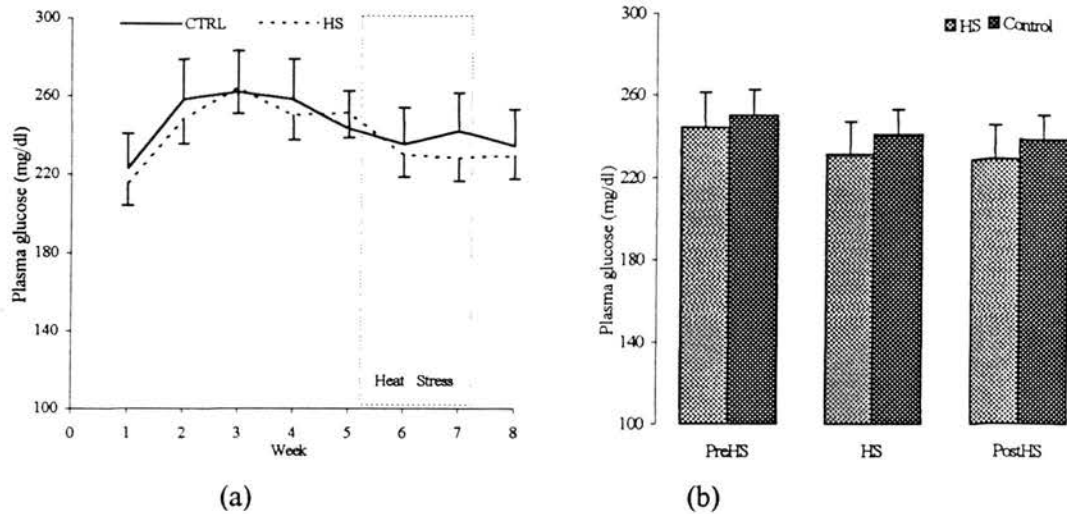


Figure 3.7. The changes in the circulating level of glucose concentration in the plasma of chronically heat stressed laying hens (32°C with 35% RH for two weeks). Heat stress treatment was imposed after the blood sampling on week 5 which was released after the blood sampling on week 7; (a) following a four week adaptation. Values represent the weekly means; and (b) plotted in a three experimental periods graph. Values represent the means within each period (\pm s.e.m.; $n=12$).

3.2. THE PATTERNS OF YOLK DEPOSITION IN THE OOCYTES DURING EXPOSURE TO HEAT STRESS

3.2.1. Introduction

The previous study (**Section 3.1**) has shown that chronic heat stress in laying hens reduces yolk size and total yolk production considerably. The studies have confirmed that heat stress reduces the yolk and egg production concomitant with reduced circulating vitellogenin and VLDL in the plasma. The reduction in precursors availability may then result in decreased uptake by the oocyte, slower yolk accumulation and oocyte development and consequent changes in egg production. Possible mechanisms of reduced yolk production include disturbances in the transport of egg yolk precursors in the blood stream and/or changes in the level of deposition these precursors into the growing oocytes in the ovary. Further experiment in this study was carried out to determine whether the mechanisms mediating reduced egg production in heat-stressed hens involved a change in the rate of yolk deposition. The present study examined the changes in egg yolk precursor levels and yolk growth in laying hens chronically exposed to high ambient temperature (35°C with 35% RH).

3.2.2. Experimental procedures

Twelve 35 week-old ISA brown laying hens were used in this experiment. They were caged individually in modules and housed in only a controlled chamber *i.e.* pre-, during and post- heat stress treatments were put in a time sequence. All the procedures (lighting pattern, egg production records, food and feeding, deep body temperature and food intake monitoring, collecting the blood samples, plasma metabolite determinations, and killing the birds at the end of the study) were similar with those in the previous study (**Section 3.1**) and as also described in the **Chapter Two: General Materials and Methods**.

The experiment was carried out for five weeks and was divided into three experimental periods *i.e.* adaptation (22°C, two weeks), heat stress (35°C, one week) and recovery (22°C, two weeks) periods all with a relative humidity (RH) 35%.

To measure the daily yolk production the birds were fed with lipophilic dyes. Two lipophilic coloured dyes (Sudan black and Scarlet R [red]) were used to colour the egg yolk materials deposited in the ovary. The two different dyes were put into different capsules for each colour. The dyes were given on alternate days (red-black-red, etc.). This technique allowed the investigation of the amount of yolk deposited in the oocyte on daily basis as previously carried out by Gilbert (1971).

The eggs were boiled to fix the coloured yolk. The colours appeared in the sectioned egg yolk clearly as spheres in three dimensions (for the details of feeding and preparation of the dyes see **Section 2.2.4.1**). Thus the sphere's volume can be calculated using the formula:

$$V = V_2^3 - V_1^3 \Rightarrow V = \left(\frac{\pi \times d_2^3}{6} \right) - \left(\frac{\pi \times d_1^3}{6} \right)$$

where V = volume; $\pi = 3.142$; d = diameter.

3.2.3. Results

3.2.3.1. Food intake and body temperature

Exposure to a high ambient temperature caused in a heat stress condition which was characterised by increased body temperature as one of thermal stress indicators. Heat stressed birds showed an increase in their body temperature ($P < 0.01$).

Similar to the result of the previous study (**Section 3.1**) heat stress reduces voluntary food intake compared to those values for the same birds during pre-heat stress (adaptation) period. Heat stressed birds have to reduce their voluntary food intake in order to reduce the heat production and avoid hyperthermia. The present study confirmed the phenomena. The reduction of food intake in heat stressed birds represented a 35% decrease compared to those values in the pre-heat stress period of the same birds, as shown in **Table 3.4**.

Table 3.4. Effects of heat stress on food intake and body temperature (weekly; $n=12$).

	Adaptation		Heat Stress		Recovery		<i>P</i>
	<i>Mean</i>	<i>s.e.m.</i>	<i>Mean</i>	<i>s.e.m.</i>	<i>Mean</i>	<i>s.e.m.</i>	
Food intake (g)	164.6 ^a (100%)	9.80	106.6 ^b (65%)	3.48	144.9 ^a (88%)	6.62	<0.05
Body temperature (°C)	41.05 ^a (100%)	0.02	42.11 ^b $\Delta=1.06$	0.09	41.28 ^a $\Delta=0.23$	0.03	<0.05

3.2.3.2. Egg production and yolk precursor concentrations

Heat stress also reduces egg production as summarised in **Table 3.5** in terms of egg number, total egg mass produced and yolk production. Birds from heat stress group were less capable of producing egg numbers (-17%), egg mass per bird (-21%), yolk mass per egg (-5.6%) and total yolk mass production (-22%) and total egg mass (-20.9%). These reductions were consistent with the previous results in this study (**Section 3.1**) although they were not numerically identical.

Table 3.5. Effects of heat stress on egg production (weekly; $n=12$).

	Adaptation	Heat Stress		Recovery	s.e.m.
\bar{x} egg weight (g)	66.0 ^a	63.1 ^a	(-4.4%)*	63.6 ^a	1.55
Σ egg numbers (n)	52 ^a	43 ^b	(-17%)	40 ^b	6.24
Σ egg mass (g)	3432 ^a	2713 ^b	(-20.9%)	2544 ^b	471.5
\bar{x} yolk mass per egg (g)	18.70 ^a	17.66 ^b	(-5.5%)*	17.81 ^a	0.56
Σ yolk mass (g)	972.4 ^a	759.4 ^b	(-22%)	712.4 ^b	138.55

Means in each column followed by different superscripts differ significantly ($P<0.05$).

* effects of heat stress.

The results of the present study showed that both plasma concentrations of vitellogenin and VLDL were significantly decreased in heat stressed birds in comparison with the values in control group; although, again, the pattern of their decrease were not similar (**Table 3.6**). During the recovery period, VLDL

concentration seemed to have a prolonged effects of heat stress whereas the vitellogenin concentration appeared returned to a normal value earlier.

Heat stress significantly decreased both vitellogenin, as zinc plasma concentration by 25% and VLDL, as plasma triglyceride by 19% concomitant with decreases in egg number (-17%), total egg mass (-21%) yolk mass per egg (-5.6%) and total yolk mass (-22%).

Table 3.6. Effects of heat stress on plasma vitellogenin and VLDL concentrations

	Day	Vitellogenin ($\mu\text{g/ml}$)		VLDL (mg/dl)	
		\bar{x}		\bar{x}	
Pre Heat Stress	1	3.51 ± 1.25		13.90 ± 2.75	
	7	3.61 ± 0.69	3.62	14.20 ± 2.69	14.31
	14	3.73 ± 0.30		14.80 ± 3.30	
Heat Stress	17	2.47 ± 0.38	2.70	12.30 ± 3.38	11.60
	21	2.92 ± 0.28		10.90 ± 2.28	
Recovery	28	3.37 ± 0.37	3.50	9.36 ± 2.37	10.11
	35	3.64 ± 0.15		10.85 ± 3.15	

3.2.3.3. Patterns of yolk deposition

The amount of yolk daily deposited in the determination of yolk growth was calculated by the methods of Gilbert (1984). Eggs from hens fed with different dyes on consecutive days were boiled to fix the coloured yolk in spheres. The volumes of the yolk deposited on a daily basis in the growing oocyte were obtained by measuring the diameter of each ring in a boiled yolk for each group. The measurements of the diameter obtained were used to yield the volume of the spheres (**Table 3.7**).

Since only one chamber was used in this trial, egg production data of the control group were the eggs collected and boiled before heat stress was imposed (pre-heat stress). The egg production data of heat stress group were based on the eggs laid and boiled at a week after heat stress treatment started.

Table 3.7. Means of the radius (mm) and volume (mm³) of yolk deposition from boiled yolk of each group on daily basis.

Day	Heat stress		Control	
	Diameter (mm)	Volume (mm ³)	Diameter (mm)	Volume (mm ³)
1	5.2	73	6.3	132
2	9.3	421	8.8	362
3	10.2	549	12.2	941
4	12.1	936	13.8	1387
5	15.1	1818	16.3	2258
6	17.9	3004	17.7	2890
7	17.8	2939	18.2	3182
8	16.3	2268	16.9	2537
9	15.1	1790	15.7	2010

The volumes deposited on a daily basis in growing oocytes were then plotted to work out the pattern of daily yolk deposition (**Figure 3.8**). The pattern showed that there were significant differences ($P<0.05$) on the days 3, 4 and 5 between two groups in daily yolk deposition. Although there was a tendency that heat stressed birds deposited less amount of yolk on daily basis on the days 7, 8 and 9 but they were not statistically significant.

The present study also confirms that application of the lipophilic dyes was effective and useful in colouring the yolk. The lipophilic dyes fixed and coloured the yolk deposited in the growing oocytes that enabled to the determination of the

amount of yolk deposited on daily basis when the different colour of dyes were fed on alternate days. The dyes form coloured concentric spheres in the yolk after boiling the egg (**Figure 3.9**).

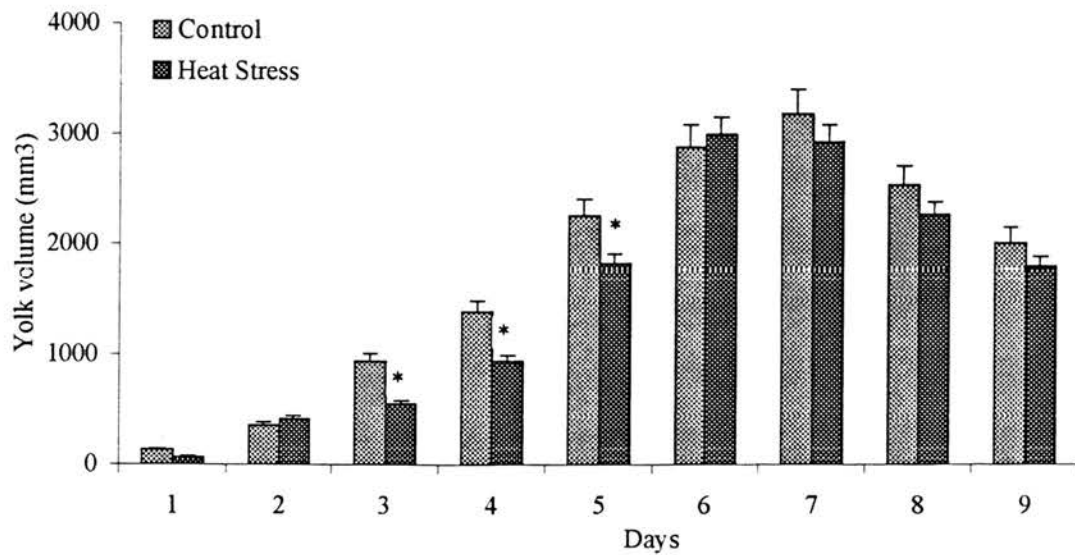


Figure 3.8. The yolk growth was determined by the daily pattern of the volume of yolk deposited in the follicles by the use of dyes to colour the yolk. The pattern of the amount of yolk deposited in follicles of heat stressed hens (35°C with 35% RH for a week) were compared to those in thermoneutral birds. Values represent the means within day production (\pm s.e.m.; $n=24$).

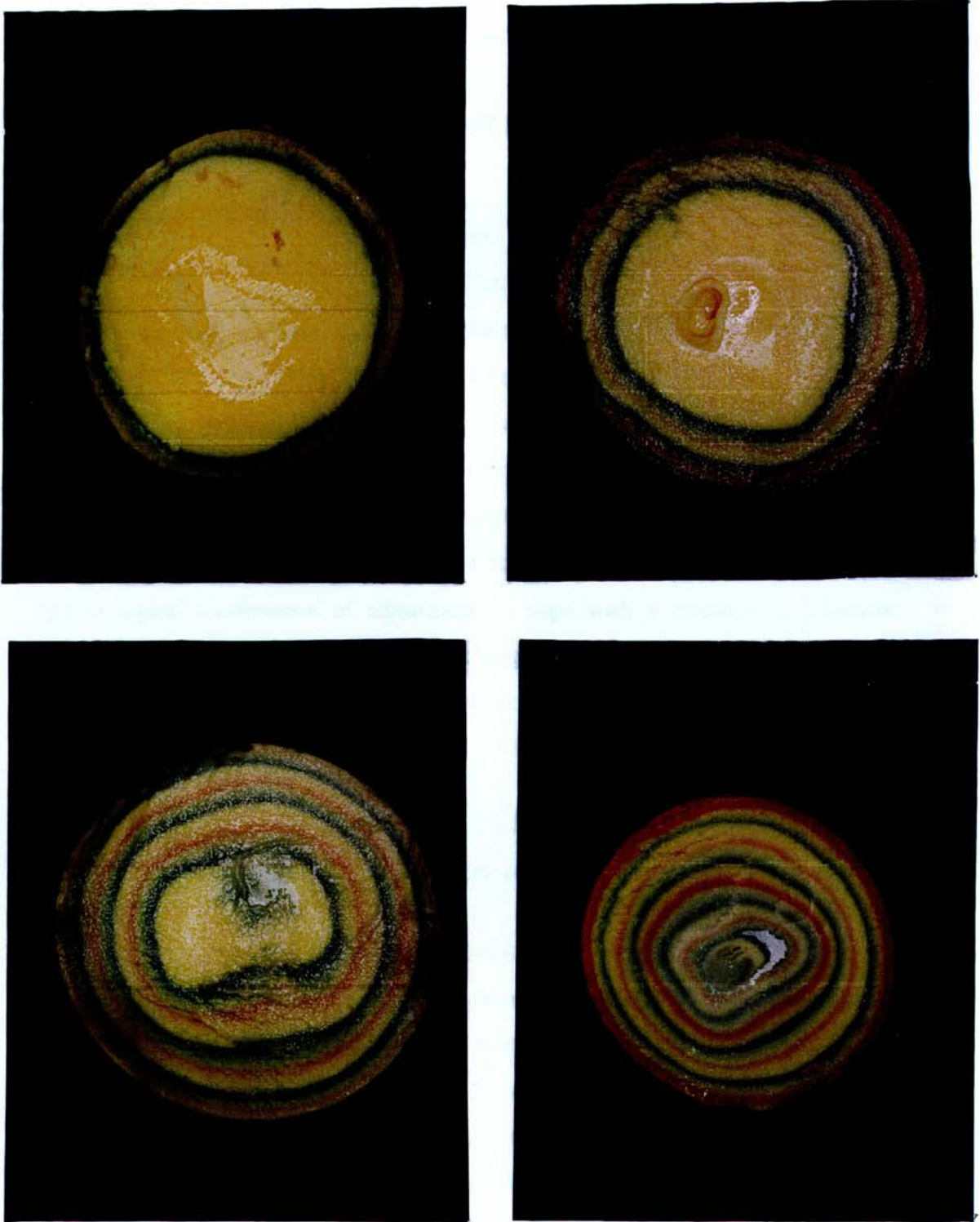


Figure 3.9. The lipophilic dyes (black and red) fixed and coloured the yolk deposited in the growing oocytes. The pictures from left to right show when dyes were fed to a hen and coloured the oocyte from two to nine days before oviposition.

3.3. DISCUSSION

3.3.1. Yolk precursors and other physiological changes during exposure to heat stress

During thermal stress, birds alter their behaviour to help maintain body temperature within the normal limits. There are some physiological and biochemical disturbances during exposure the laying hen to high ambient temperatures. A number of reports have shown that respiratory alkalosis occurs when chickens are subjected to high ambient temperatures (Darre *et al.*, 1980; Odom *et al.*, 1982; Arad and Marder, 1983; Odom *et al.*, 1986; Beers *et al.*, 1989; Odom and Ono, 1991; Koelkebeck and Odom, 1994). The present results agree with these previous reports. The losses in production observed due to heat stress are the results of complex physiological mechanisms of adjustment to cope with a stressful environment. In addition, reproductive adaptations to changes in environmental conditions may be characterised by measuring changes in vitellogenin production as an index of rates of egg yolk precursor synthesis and secretion (Mitchell and Carlisle, 1991).

Changes in deep body temperature and food intake

During exposure to high ambient temperature respiratory changes are observed in addition to hormonal and circulatory changes. The physiological responses to heat stress in birds involve the functional integration of several organs to meet the metabolic needs of birds that are trying to dissipate heat. Radiant and conductive heat loss is dispersed through anatomical specialisation in birds. This heat dissipation, basically occurred in the featherless zones (by raising wings), involves the regulation of the blood flow through the arterio-venous network acting as a heat exchanger (Midtgard, 1989). Another effect of heat stress was the accumulation of heat that could not be dissipated which in turn causes an increase of deep body temperature. Changes in the body temperature of heat stressed bird in this study are shown in **Table 3.1.**

There was also an immediate increase in water consumption as the birds experiencing a heat stress (although the study did not measure water consumption). Increased water consumption is to assist the process of evaporative cooling from respiratory surfaces (Smith and Oliver, 1971; May and Lott, 1992). This may partly contribute in the reduced food intake as the birds have less time (and space in the gastro-intestine?) for the food besides they physiologically try to reduce their food intake (heat production) and avoid hyperthermia. The changes of food intake of this study are shown in **Table 3.2**.

Suppression on food intake to avoid hyperthermia during exposure to high ambient temperature is a recognised thermoregulatory strategy in the domestic fowl (Mitchell and MacLeod, 1983). The decline of voluntary food consumption reduces the contribution of metabolic heat to the total heat load that requires dispersion (Otten *et al.*, 1989). In addition, heat stress would encourage a reduction in energy expenditure (MacLeod *et al.*, 1982, 1988).

Egg production

The results of this study show that heat stress over a two week period causes a significant detrimental effect on egg production; both the number and weight of eggs which results in a substantial decrease in egg production (**Table 3.3**). In the comparison of the mean egg weight (-8%) and the mean egg weight per day, representing respectively the mean of egg weight (-8%) produced and the theoretical mean egg weight if they had to be laid daily. Heat stress decreased egg yolk size (-4%) and total yolk production (-37%) then these phenomena may be reflected in the synthesis and/or levels of the egg yolk precursors in the circulation. The concentrations of plasma vitellogenin and VLDL decreased by 34% and 29% respectively, although in this case the reduction in VLDL was less severe compared with those in vitellogenin.

There were a 8% reduction in the mean of egg weight and a 37% yolk produced by heat stressed birds. In a simple way a decreased egg production can be said as the bird producing eggs in a slower rate than a normal time birds can do. This can be

assumed that heat stressed birds required more time to produce an egg. This changes in the time of producing an egg in heat stressed birds may be due to the less availability of egg yolk precursors in the circulation and/or a disruption in the deposition process of these materials into the growing oocytes in heat stressed birds, besides changes caused by reduced food intake. However, the reduction of voluntary food intake in this study was 28% whilst total yolk, as the most important part of an egg, production decreased by 37%. This may lead to an assumption that other physiological changes occurred during exposure to high ambient temperature in addition to changes caused by reduced food intake.

Comparison between mean yolk weight and mean yolk weight per day showed the same response as for egg production. However, the difference between the two groups for the mean yolk weight per day represented a 35% reduction. It thus appears that exposure to heat stress reduces the number of eggs laid, the size or weight of each egg produced and the total yolk production and yolk per egg. The appearance of reduced egg production in this study was concomitant with reduced the availability of egg yolk precursors (vitellogenin and VLDL) in the circulation to which it may be attributable.

Plasma egg yolk precursor concentrations

Yolk precursors are synthesised in the liver of the laying hen and transported in the plasma to the ovary where they are taken up into the developing follicles by receptor-mediated endocytosis (Griffin, 1992). The study confirmed the method in determinations of egg yolk precursors which was developed and described by Mitchell and Carlisle (1991) was appropriate and useful. The technique shows that vitellogenin levels measured as plasma zinc exhibit patterns similar to those of production of egg yolk precursors during sexual maturation as determined by other methods. The high degree of correlation of total plasma zinc and in particular lipoprotein associated zinc with the organic phosphorus content of the lipoprotein fraction suggest that zinc concentration is an accurate index of vitellogenin content (see details on **Section 2.4.1 of Chapter Two: General Materials and Methods**).

As can be seen in **Figure 3.1**, before the start of chronic heat stress treatment, there was no difference in plasma zinc concentration between the two experimental groups (during five weeks pre-heat stress). However, over the subsequent two weeks of treatment there was an overall significant reduction ($P<0.01$) in plasma zinc concentration in the chronically heat stressed group, representing a 36% reduction in zinc concentration (**Figure 3.1**).

Slightly different responses were observed in plasma triglyceride concentration during exposure to high ambient temperature. Unlike plasma zinc concentration, there was a slight decrease in triglyceride concentration but this was not significantly different between the two groups in the first week of heat stress. Indeed, whilst the changes in plasma triglyceride did not reach significance in the first week, the heat stress induced depression in plasma vitellogenin zinc did. (**Figure 3.1a**). A significant difference in vitellogenin concentration was also observed in the second week of chronic heat stress exposure in these groups ($P<0.05$). However, over the two weeks of treatment there was a statistically significant decrease (35%, $P<0.01$) in plasma triglyceride concentration in the chronically heat stressed group.

The results showed that both concentrations of vitellogenin and VLDL fell during heat stress. Although, the pattern of responses for each precursor altered with the period of heat stress. **Figure 3.1** shows that as VLDL concentration fell in the second week of heat stress, whilst vitellogenin concentration seemed to initially fall during the first week.

The plasma of laying hens contains high concentrations of triglyceride-rich lipoproteins. These are very similar to the yolk triglyceride-rich lipoproteins and this strongly suggests there is a direct transfer of lipoproteins from plasma to yolk (Bacon *et al.*, 1973; Christie and Moore, 1972). This may lead to a assumption that reduction in the availability of this precursor in the circulation may also be responsible for the decreased egg production in hens when they are exposed to high ambient temperature.

The two major egg yolk precursors (vitellogenin and VLDL), which are synthesised by the liver, are the main factors required for egg yolk formation. The

availability of these precursors in the plasma is vital for the growing oocytes. Any conditions causing a reduction in their availability may lead to disturbances in egg production. The findings of this study could suggest that the two yolk precursors perform slightly different response characteristics to heat stress.

It may be proposed that heat stress may inhibit yolk precursor synthesis in the liver either (1) by reduction in oestradiol secretion and/or (2) by reducing the hepatocyte receptor's sensitivity to oestrogen stimulation. Decreased precursors synthesis would account for the apparent reduction in plasma concentrations of vitellogenin and VLDL. The reduction in precursors availability may then result in decreased uptake by the oocyte, slower yolk accumulation and oocyte development and consequent changes in egg production. Although Mitchell *et al.* (1995) found that there is an adaptation in the intestinal level of heat stressed broiler in hexose uptake, the adaptive responses in the oocyte uptake mechanisms -which may occur concurrently during heat stress- have not to be characterised yet

Total and ionised calcium concentrations

Dietary calcium is ingested, stored in the crop, ground in the gizzard, and dissolved in the acidic environment of the proventriculus. Calcium ions (Ca^{2+}) pass to the upper regions of the small intestine where they are transferred into blood. From the vascular system, Ca^{2+} can be utilised directly in shell formation or stored in either the medullary or cortical bone (Etches, 1987). The storage depots of calcium in bone develop under the influence of oestrogen stimulation as the concentration of this hormone rises at sexual maturity. The depots, which are composed of a labile form of calcium phosphate termed medullary bone, are located primarily in the long bones (Etches 1996).

Heat stress may affect other aspects of bone metabolism and calcium mobilisation including the activity of alkaline phosphatase. This enzyme, which is involved in the calcium bone turnover, may reflect calcium and helps regulate the supply of adequate calcium required for the calcification of the shell in oviduct. Elevated bone reabsorption would lead to a severe osteoporosis due to lack of

calcium in the bone. Reduced availability of calcium in the blood circulation may also be contributable to the decreased egg production and this may be reflected in the lower total shell mass produced in heat stressed group (-34%; **Table 3.3**).

Miller and Sunde (1975) suggested that calcium metabolism in heat stressed laying hens may be impaired by a reduction in thyroid hormone and calcitonin production and secretion in such a way that they are maintained in balance but at lower concentrations. Similar finding was reported by Whitehead (1992); he found a reduction in plasma calcium and zinc in the laying hen when they are exposed to high ambient temperature.

Blood pH

During exposure to high ambient temperature, heat stressed birds try to dissipate the excessive heat in an attempt to maintain physiological homeostasis (Lustick, 1983). Heat dissipation by evaporative cooling at the surfaces of the mouth and respiratory passageways leads to a range of other physiological changes. Thermal hyperventilation, accompanied by severe respiratory alkalosis, can be a harmful threat, affecting the normal activity of the peripheral and central nervous system (Maskrey, 1984; Kasemi and Johnson, 1986).

Panting is one of the visible responses of poultry during exposure to heat. It was demonstrated that when body temperature of hens reaches 42°C, the panting process is initiated (Hillman *et al.*, 1985). But as panting increases the loss of carbon dioxide from the lungs, the concentrations of carbon dioxide (Wang *et al.*, 1989), and ultimately bicarbonate are reduced in blood plasma. This mechanism initially results in a plasma pH rise (Richards, 1970), which is generally referred to as alkalosis. In layers, panting, through the reduction in plasma concentrations of bicarbonate, compromises the shell gland buffering formation of CaCO_3 and thus eggshell quality (Mongin, 1968).

It is well documented that heat stress affects the blood acid-base balance of poultry by decreasing the partial pressure of arterial blood carbon dioxide (P_{CO_2}) which causes in increased blood pH (Odom *et al.*, 1982; Arad and Marder, 1983:

Bottje and Harrison, 1985; Koelkebeck and Odom, 1994). In acute or short term heat stress a marked respiratory alkalosis occurs. This is a consequence of averse ventilation during thermal polypnea or panting which increases the elimination of carbon dioxide. The reduction in the partial pressure of carbon dioxide (P_{CO_2}) in the blood therefore result in a “hypocapnic alkalosis”. This occurs in the ratio of base to acid or bicarbonate (HCO_3^-) to carbon dioxide (CO_2) has been altered. Blood pH can be described by the common Henderson-Hasselboch equation:

$$pH = pK_a + \log \frac{base}{acid} \quad \text{or} \quad pH = pK_a + \log \frac{HCO_3^-}{CO_2}$$

In chronic heat stress a further adaptation may occur which tends to restore blood pH towards normal. This involves increased excretion of bicarbonate at the kidney such that the base and acid ratio is returned towards its normal value. This does however mean that body content of both CO_2 and HCO_3^- are depleted in the chronically heat stressed and adapted bird. Disturbances in blood gas and acid-base balance and availability or disposition of HCO_3^- have detrimental effects upon egg shell production and quality. Chronic panting or heat stress induced alkalosis induces major changes in egg output and shell strength. [A simple possible explanation on changes in the acid-base balance during heat stress might be suggested as in **Figure 3.10**]. The possibility of the occurrence of tissue damage in the diagram is proposed possibly by the release of creatine kinase iso-enzyme to the plasma (Mitchell and Sandercock, 1995).

This problem or respiratory alkalosis can be combated nutritionally by providing a source of anion via feed or water. For example, Teeter and Smith (1986) have shown that supplemental aluminium chloride in drinking water of chronically heat stressed birds can return blood pH to normal and enhance production. In addition, the provision of ammonium chloride (Branton, *et al.*, 1986) or carbonated water during acute heat stress (Bottje and Harrison, 1985) has been found to decrease blood pH.

Plasma inorganic phosphorus concentration

The normal function of tissues are dependent upon the stability of the total osmolarity of intracellular and extracellular fluids. The major ions of the plasma are sodium, chloride, potassium, calcium, phosphate, sulphate and magnesium. The concentrations of the major ions are also important in determining the pH of the body fluids (Etches, *et al.*, 1995). High ambient temperature has been associated with increased plasma sodium and chloride but decreased plasma potassium and phosphate (Ait-Boulahsen *et al.*, 1989).

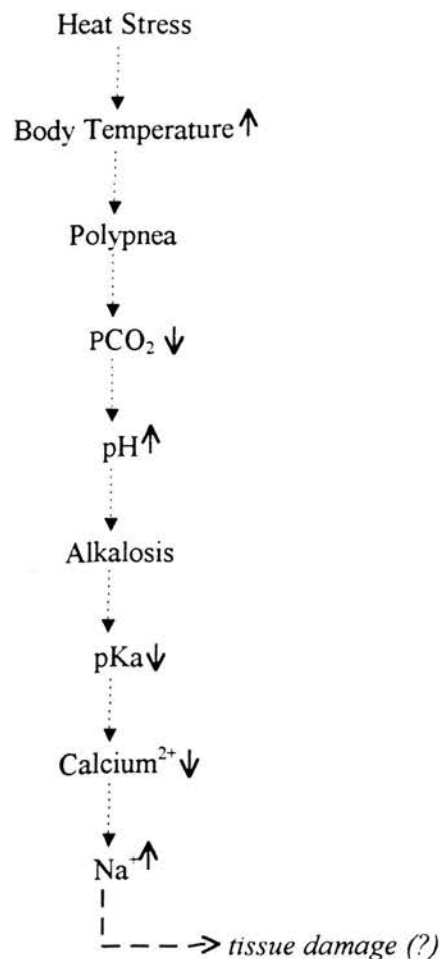


Figure 3.10. Diagram of possible physiological responses to exposure to high ambient temperature which in turns may lead to a tissue damage.

Similar findings were found in the present study which showed that heat stress reduces the plasma concentration of inorganic phosphorus (**Figure 3.6**). In normally hydrated fowls, heat stress (35-45°C for 10-12 h) produced no significant changes in the serum concentrations of sodium, potassium, chloride and calcium, or in serum osmolality, although serum phosphate declined (Arad *et al.*, 1983). However, Wang *et al.* (1989) reported that heat stress (37°C, 45% RH for 150 min) did not significantly alter plasma osmolality. The different responses may be attributed to the differences in the extent and duration of heat exposure, and to the fact that birds used by Arad *et al.* (1983) were acclimatised to high ambient temperature for long period prior to the experiment.

The concentration of phosphorus in the circulation is related to calcium. Under normal physiological conditions, the concentrations of both minerals are balanced. Garlich and McCormick (1981) reported that calcium and phosphorus balance seems to have an effect on survival time during periods of acute heat stress. Their finding showed a direct relationship between plasma phosphorus and survival time, and an inverse relationship with plasma calcium.

Plasma glucose concentration

No changes in plasma glucose concentration were induced by the chronic heat stress in this study which confirms the similar result reported by Mitchell and MacLeod (1993). This homeostasis might be expected as chickens do maintain steady glucose levels in a number of different conditions. The lack of difference in plasma glucose levels between groups during exposure to high ambient temperature suggests that there was only a little effect on the plasma metabolite concentration induced by any changes in water balance due to warm environment. It may be suggested that changes in carbohydrate metabolism, energy resources and plasma glucose do not play an important role in affecting the synthesis of egg yolk precursors in the liver and thus their circulating levels.

3.3.2. The patterns of yolk deposition in the oocytes during exposure to heat stress

The results of the present study show that exposure to high ambient temperature caused similar heat stress-induced physiological responses as described in the previous section (3.3.1). These include behavioural changes such as panting, raising wings, drinking more water, and reducing food intake whilst the birds experiencing hyperthermia.

There is no doubt that high ambient temperatures impose limitations on the performance of both broilers and laying hens that may be related to food intake. However, only part of the impairment in laying performance might be due to reduced food intake. Smith and Oliver (1972) showed that 40-50% of the reduction in egg production and egg weight at 38°C is due to reduced food intake. It means that reduced egg productions in heat stressed birds are not only due to reduced food intake. In addition, the responses of birds subject to cyclic high ambient temperature are not too different from that due to constant temperature (Daghir, 1995).

Also taking into account that the egg production in this study decreased more than the decrease in egg yolk precursors, this may lead to the suggestion that there is a disturbance in the transfer of these precursors to the developing oocytes. The work in the **Section 3.2.** determined the daily volumes deposited in growing oocytes and plotted to work out the pattern of daily yolk deposition (**Figure 3.8**). The pattern showed that there was a significant difference between two groups in daily yolk deposition. Heat stress caused significant numerical reductions in the yolk volume accumulation on days 3, 4 and 5 only, but failed to reach significant differences for the accumulation on the remaining days.

There must be some other physiological disturbances and mechanism changes during exposure the hen to high ambient temperature.

Exposure to high ambient temperature is also associated with a decline in blood-pressure, an increase in cardiac output and a decrease in peripheral resistance (Sturkie, 1976; Darre and Harrison, 1987). As birds become acclimatised to elevated ambient temperature, however, cardiac output decreases, blood pressure increases

and peripheral resistance returns to normal. However, there was a variation among the results in heart rate of chickens following exposure to heat stress. Darre and Harrison (1987) suggested that the variation may be, partly, not only due to the differences of heat load employed, but may also be the consequence of the trauma of repeated blood sampling especially in the acute heat stress studies) which increases heart rate, overriding the inhibitory effect of thermal stress. Blood flow from the body core to the periphery plays a significant role in the transfer of heat in the deep body tissues to the peripheral tissues that are capable of dissipating heat to the surrounding environment (see Dare and Harrison, 1987). During acute heat stress, the cardiovascular system distributes blood to functions related to thermoregulation, giving only secondary importance to other functions. In heat exposed hens, for example, Bottje and Harrison (1985) demonstrated that blood flow to the viscera was reduced by 44%. It is likely that there is also reduction of blood flow to the reproductive organs included the ovary which may lead to the reduction in the amount of yolk precursors can be deposited to the growing oocytes. This condition could be worst when the availability of the yolk precursors is reduced during exposure to high ambient temperature as shown in the previous section. Apart of the effect of the decreased food intake, there are some possible mechanisms involved whether the decreased yolk precursors in the circulation is due to decrease in the oestrogen concentration which in turn fails to trigger to normal synthesis and/or reducing the receptor's ability in the liver to bind oestrogen.

It is also indicated that heat stress has effects on reproductive performance and its hormones. Decreased egg production in heat stressed hens is suspected to at least partly influenced by the ovulatory hormones as reported by Donoghue *et al.* (1989) and Novero *et al.* (1991). They found that heat stress reduces serum luteinising hormone (LH) levels, hypothalamic content of luteinising hormone-releasing hormone (LHRH) and the preovulatory surges of LH and progesterone. Since the preovulatory surges of LH and progesterone are controlled in a positive feedback loop (Etches and Cunningham, 1976; Williams and Sharp, 1978; Johnson *et al.*, 1985) and since both hormone levels are depressed concomitantly during heat stress (Novero *et al.*, 1991), it is difficult to identify the site(s) of action of heat stress.

Since heat stress is related to the changes of metabolic rate, thyroid hormones also play an important role in adaptation to heat stress. In chickens, although still crucial, thyroid hormone secretion is depressed as ambient temperature increases and heat tolerance improves as thyroid function is reduced (Fox, 1980; May, 1982; Bowen *et al.*, 1984). The two active forms thyroid hormones are T4 and T3, and the inactive form is reverse triiodothyronine (r-T3). The selective peripheral conversion of T4 to T3 or r-T3 is believed to play an important role in thermoregulation in domestic fowl (Decuypere *et al.*, 1980; Rudas and Pethes, 1984); when chickens are exposed to warm environment, T4 is inactivated by conversion to r-T3, whereas during cold exposure T4 is converted into T3, which stimulates metabolic rate. While it is generally accepted that T3 stimulates metabolic rate and both T3 and T4 are depressed following heat stress, this pattern is not universally accepted. For example, plasma T3 and T4 concentrations have been reported to increase, decrease or remain unchanged following heat stress in Japanese quail and pigeons (Bobek *et al.*, 1980; Boven and Washburn, 1985; Pilo *et al.*, 1985). This leads to conclusion that the complex physiological response to heat stress does not consistently affect plasma concentrations of thyroid hormones (Daghir, 1995).

The present study confirms that application of the lipophilic dyes is appropriate for measurement of yolk deposition as clearly shown in **Figure 3.9**. It can also be suggested that exposure to an increased ambient temperature alters the pattern of yolk deposition in the oocyte in laying hens.

CHAPTER FOUR

THE EFFECTS OF OESTROGEN ON PLASMA YOLK PRECURSOR LEVELS AND EGG PRODUCTION IN HEAT STRESSED HENS

4.0. Introduction

The results of previous studies (**Chapter Three**) showed that chronic heat stress significantly decreased egg production concomitant with reduction in plasma yolk precursor concentrations. Ovulation in a laying hen is maintained by regular increases in the plasma concentrations of luteinising hormone (LH), progesterone, testosterone and oestradiol-17 β (Etches and Cunningham, 1977; William and Sharp, 1988). It is therefore necessary to evaluate the role of oestrogen in laying performance and yolk precursor concentrations in heat stressed hens.

The study in this **Chapter Four** examines the effects of oestrogen administration *in vivo* on plasma yolk precursor concentrations and egg production in laying hen chronically exposed to high ambient temperatures. This study consists of two sections. First section evaluates the influence of daily administration of oestrogen (as oestradiol benzoate-17 β at a 2.5 mg/kg body weight) on egg plasma yolk precursors and egg production in 50 weeks old laying hens. The hens employed in the first trial were at the peak of laying period. The second section examines the effects of oestrogen *in vivo*, with the same dose of 2.5 mg/kg body weight also in daily basis, on plasma egg yolk precursors and egg production in heat stressed laying hens of 80 weeks old of age. In the second trial of this study the birds used represented hens in the end of laying period. The results of both experiments were discussed and brought together in a comparison with those value in hens at thermoneutral temperature. Therefore, the results of those trials can be applied to investigate whether oestrogen induces similar responses on plasma egg yolk precursors and egg production in the different stages of lay of heat stressed hens.

Although reduced food intake is attributable to the decreased egg production in heat stressed hens, this phenomenon could possibly also due to hormonal changes and/or disturbances in the hen's cycle process of egg production. Taking into account that the production of an egg is a complex physiological process and any hormonal or ovulatory changes caused by any stress can lead to changes in egg production. The decreased food intake and effects of chronic exposure to heat stress probably alters oestradiol secretion and/or reduced sensitivity of the hepatocytes to oestrogen stimulation. These studies were not only designed to investigate the role of exogenous oestrogen (as oestradiol benzoate, *in vivo* administration) on changes in egg yolk precursor concentrations and egg production, but also to examine whether liver is still capable of synthesising vitellogenin and VLDL during heat stress.

4.1. Effects of oestrogen in heat stressed hens at peak- and end of lay

In this section the changes of plasma levels of egg yolk precursor and egg production were examined in heat stressed birds and the values compared to those reared in thermoneutral environment for both groups receiving either oestrogen or vehicle (as control) treatment. This section consists of two experiments which used different birds at different ages. **Experiment 1** employed a temperature of 30°C with 80% RH as the elevated environment on 50 weeks old hens while **Experiment 2** employed 35°C with 55% RH and used 80 weeks old hens. All experiments employed a chronic heat stress for two weeks and oestrogen treatments were given at the second week of heat stress period on consecutive day basis for seven days.

4.1.1. Experimental procedures

4.1.1.1. Experiment 1

Twenty-four laying hens, 50 weeks old Hi-Sex, were used in this study. The birds were randomly assigned to four groups ($n=6$) and receiving oestrogen (E_2) or

vehicle (Veh) treatment in two different climate chambers. All birds were kept for two weeks for adaptation period at 21°C with 35%RH. Heat stressed (HS) groups were kept in a climate chamber at 30°C with 80% RH for two weeks, whilst control group was maintained at thermoneutral (TN) temperature in the control chamber. Birds in all groups had access to food and water *ad libitum*. After a week of heat stress treatment, the hens in control groups (TN Veh and HS Veh) were injected subcutaneously with vehicle alone (olive oil), whilst the oestrogen groups (TN E₂ and HS E₂) were subcutaneously injected with oestradiol-17 β at a dose of 2.5mg/kg body weight on consecutive daily basis for a week. The hens in control groups (TN Veh and HS Veh) received vehicle (olive oil) alone. The grouping and treatments were as illustrated in the **Table 4.1**. Blood samples (in **Figures 4.2** and **4.3**) were taken during (1) the period of adaptation, as the baseline before applying the treatments (day 4), (2) in the first week of heat stress (day 8 and 12) and administration of oestrogen (day 16 and 20), and also (3) whilst in the recovery period (day 24 and 28).

Table 4.1. Grouping of hens ($n=6$) indicating room temperatures with humidities and treatments.

Group	Chamber	Environmental condition	Treatment
TN Veh	1	21°C and 50 \pm 5% RH	Vehicle injection (olive oil)
TN E ₂	1	21°C and 50 \pm 5% RH	Oestrogen (2.5 mg/kg BW)
HS Veh	2	30°C and 80 \pm 5% RH	Vehicle injection (olive oil)
HS E ₂	2	30°C and 80 \pm 5% RH	Oestrogen (2.5 mg/kg BW)

TN= thermoneutral, Veh= vehicle, E₂= oestrogen, HS= heat stress

4.1.1.2. Experiment 2

In this trial fourteen Hi-Sex hens, 80 weeks old at almost the end of lay, were used. Similar to the procedures of the previous section, all birds were kept individually with access to food and water *ad libitum*. The hens used in the trial were selected among

those with egg production records, and only hens which still laid everyday were selected for the study. The birds were then divided in two groups, one experimental group and the other one as control group. The birds from both groups, seven birds each, were kept in a controlled climate chamber with a lighting pattern of 14L:10D under a thermoneutral condition (22°C with 50% RH) for two weeks. This condition was then followed by a two week heat stress exposure (35°C with 55% RH) for both groups. Similar to the **Experiment 1**, after a week of heat stress treatment the birds (treatment group) were injected subcutaneously with oestradiol-17 β at a dose of 2.5mg/kg body weight on a consecutive daily basis for a week whilst control birds received vehicle (olive oil) alone. Blood samplings were taken once in the adaptation period, during the first week of heat stress treatment and during oestrogen treatment when heat stress was still imposed.

4.1.2. Statistics Analysis

These two experiments conducted in this study were different in their experimental designs. **Experiment 1** had a more appropriate control groups which were kept under thermoneutral condition throughout the experimental period, which meant each treatment (heat stress or oestrogen) had its own control group. Whilst in the **Experiment 2** both two groups were put in a chamber which the only different treatment was the injection, *i.e.* oestrogen or vehicle. All groups in each experiment were tested across before each treatment to examine whether there was a difference before and after applying treatments by the use of Anova (balanced Anova or General Linear Model - GLM). Student's *t*-Test was applied for comparison of two groups.

4.2. RESULTS

4.2.1. Results of Experiment 1

The effects of heat stress and oestrogen treatments on body temperature, yolk production and yolk precursors, triiodothyronine, thyroxine, and egg production are summarised in **Figures 4.1 - 4.5** and **Tables 4.2 and 4.3**.

Consistent to the results in the previous studies (**Chapter Three**) heat stress in this trial also caused in increased body temperature and reduced food intake as shown in **Tables 4.2** and **4.3**.

Table 4.2. Effects of heat stress and oestrogen on body temperature ($n=6$).

Week	Body temperature ($^{\circ}\text{C}$)				Pooled s.e.m	<i>P</i>
	TN Veh	TN E_2	HS Veh	HS E_2		
0	40.9	41.0	40.8	40.9	0.07	NS
1	40.8	41.1	41.8 $\Delta^* = 1.0^{\circ}\text{C}$	42.1 $\Delta = 1.2^{\circ}\text{C}$	0.04	<0.01
2	41.0	41.2	42.0 $\Delta = 1.2^{\circ}\text{C}$	42.2 $\Delta = 1.3^{\circ}\text{C}$	0.03	<0.01

Week 0= thermoneutral (TN); week 1 = heat stress (HS); week 2= heat stress and oestrogen (E_2) treatment; Veh= vehicle (olive oil injection as control).

* thermal difference due to heat stress

Table 4.3. Effects of heat stress on food intake of laying hens ($n=6$).

Week	Food intake (g)				Pooled s.e.m	<i>P</i>
	TN Veh	TN E_2	HS Veh	HS E_2		
0	131.6	119.7	123.7	124.4	5.87	NS
1	122.4	112.9	89.9	85.4	9.35	<0.01
2	127.3	123.5	91.8	89.2	8.73	<0.01

Week 0= thermoneutral (TN); week 1= heat stress (HS); week 2= heat stress and oestrogen (E_2) treatments; Veh= vehicle (olive oil injection as control).

The changes of egg production before (week 0), during heat stress treatment (week 1) and during both treatments of heat stress and oestrogen (week 2) are listed in **Table 4.4**. Similar with the results in previous studies (**Chapter Three**) heat stress in this trial decreased egg number by 18% and 44% in week 1 and 2 respectively (HS Veh group). Whilst in heat stressed-oestrogen treated group (HS E_2) heat stress decreased egg number by 23% in week 1 although was only 26% reduction in week 2.

In terms of total yolk mass production in week 1, heat stress caused 20% and 24% reduction in heat stress groups respectively (HS Veh and HS E₂ groups). This reduction became higher (44% and 22% reductions) respectively in week 2 when the oestrogen treatment was employed to the heat stressed hens. Similar results were also found in the total egg mass produced in these groups. Oestrogen increased total yolk mass produced and egg mass in hens kept under thermoneutral conditions by 14%. Oestrogen significantly improved yolk size in both heat stress and thermoneutral groups (**Figure 4.1**).

Table 4.4. Egg production of treated groups in comparison with control group ($n=6$).

		TN Veh	TN E ₂	HS Veh	HS E ₂	s.e.m.
Egg Number (n)	Week 0	39	40	38	39	0.82
	1	41	39	31	30	5.56
	2	38	42	21	29	9.40
\bar{x} yolk weight (g)	0	17.9	17.3	17.8	17.5	0.28
	1	18.0	18.2	17.3	17.2	0.50
	2	18.2	18.9	18.0	18.3	0.39
\bar{x} egg weight (g)	0	52.8	53.5	57.9	53.3	2.37
	1	55.1	58.0	54.6	57.2	1.63
	2	56.1	57.4	53.3	57.5	1.96
Σ yolk mass (g)	0	698.1	693.8	674.9	681.9	10.67
	1	739.6	708.9	537.7	516.2	14.93
	2	690.7	792.6	378.8	531.2	81.63
Σ egg mass (g)	0	2059.2	2140.0	2200.2	2078.7	63.86
	1	2259.1	2262.0	1692.6	1716.0	21.30
	2	2131.8	2410.8	1119.3	1667.5	65.65

Week 0= adaptation, 1= heat stress treatment; and 2= heat stress and oestrogen treatments.

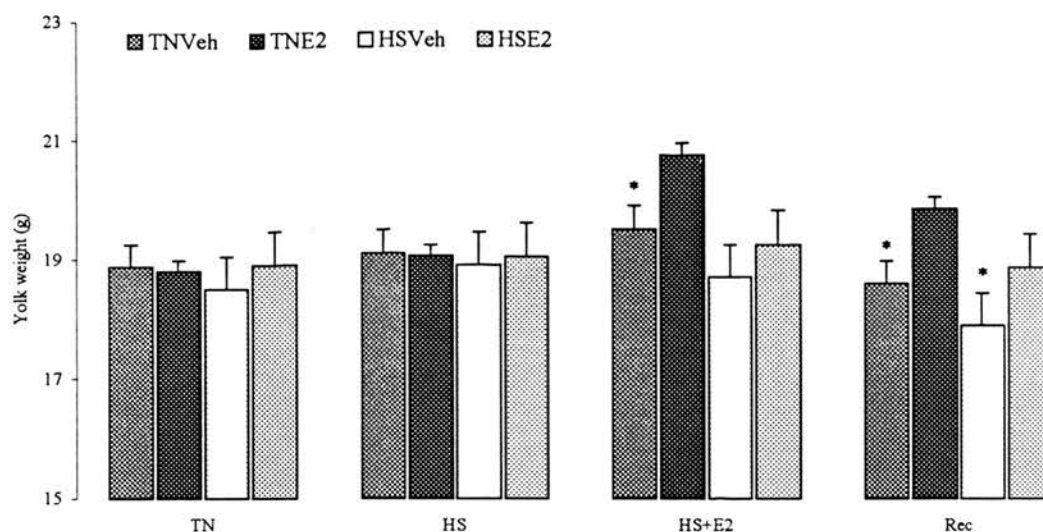


Figure 4.1. Yolk mass produced by hens in both thermoneutral and heat stress conditions (means+s.e.m.).

Oestrogen did affect the egg yolk precursor concentrations in both thermoneutral and heat stress groups as can be seen in **Figure 4.2**. Heat stressed hens showed similar responses to exogenous oestrogen stimulation in comparison to thermoneutral birds in changes in vitellogenin and VLDL concentrations (**Figure 4.3**). It is likely that during exposure to high ambient temperature the heat stressed birds showed similar responses to oestrogen stimulation compared to those birds kept under thermoneutral environment. This conditions were true for both VLDL and vitellogenin changes.

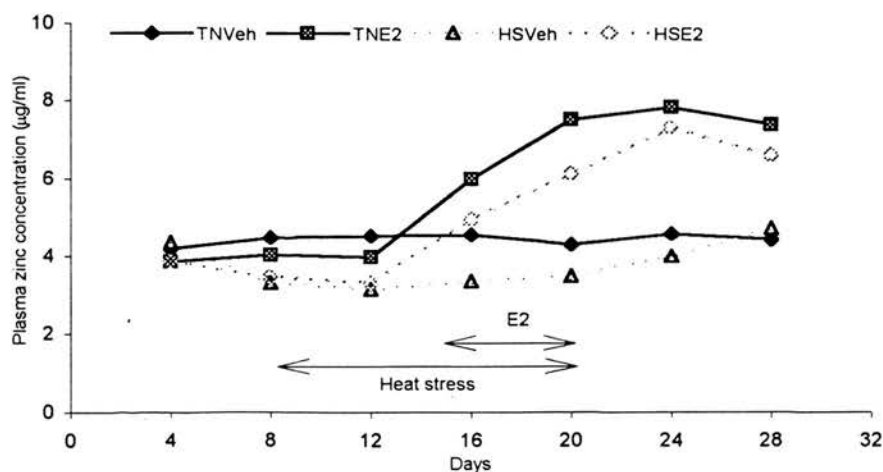


Figure 4.2. Vitellogenin concentration changes during exposure to heat stress and oestrogen (E_2) treatments periods. TN= Thermo-neutral; Veh= vehicle (control); HS= heat stress. Plasma vitellogenin was measured as plasma zinc concentration.

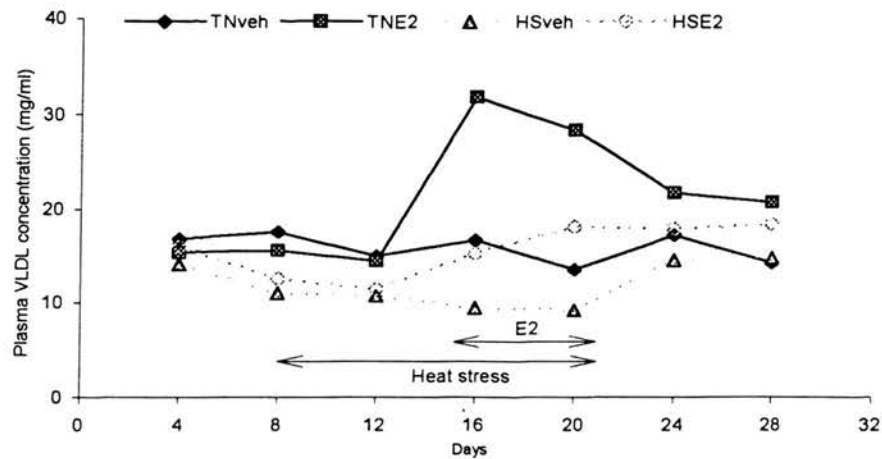


Figure 4.3. Triglyceride-rich VLDL concentration changes during exposure to heat stress and oestrogen treatment periods. TN= Thermoneutral; Veh= vehicle (control); HS= heat stress. Plasma VLDL was measured as triglyceride concentration.

Oestrogen treatment did not affect plasma thyroxine concentrations in both thermoneutral and heat stress groups as showed in **Figure 4.4**, but did have a significant effect on plasma triiodothyronine concentration (**Figure 4.5**). This may be explained that there was an increase in the deiodination of thyroxine which in turn resulting in the increased triiodothyronine as an active form of thyroid hormone. Taking into account that the major source of triiodothyronine is from the conversion of thyroxine.

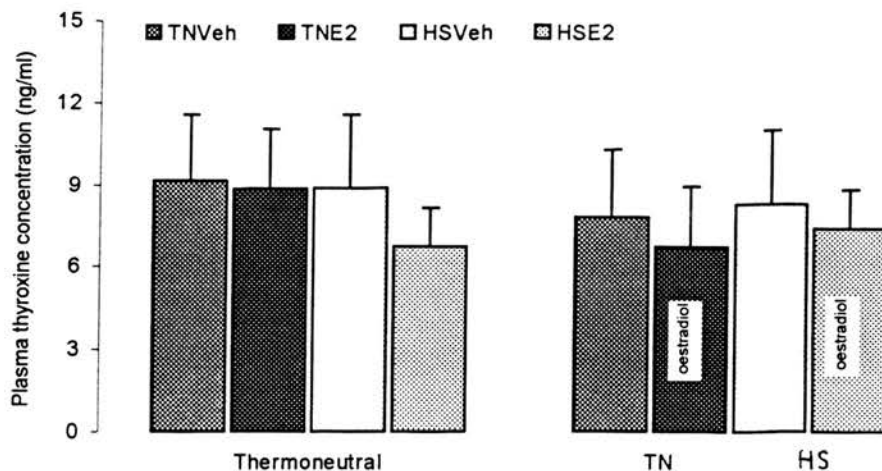


Figure 4.4. Plasma thyroxine concentrations of laying hens in both thermoneutral and heat stress and oestrogen (E_2) treatment groups. TN= thermoneutral; Veh= vehicle (control); HS= heat stress (means + s.e.m.; ; $n=6$).

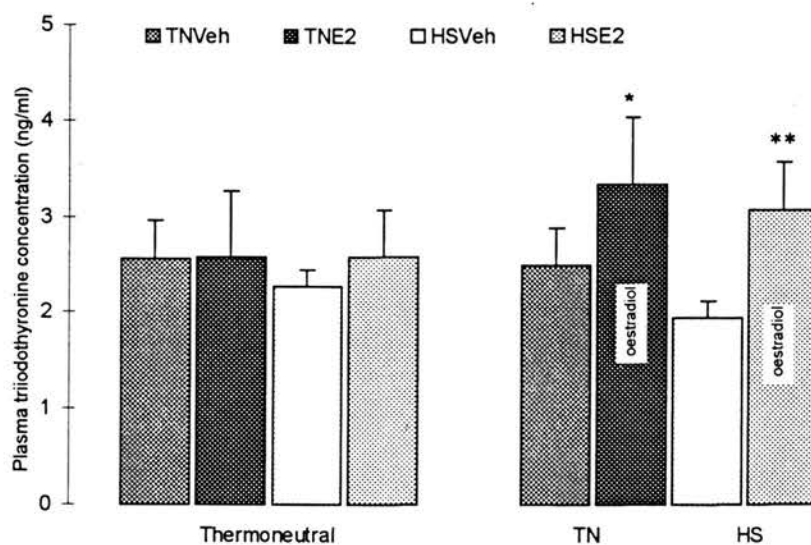


Figure 4.5. Plasma triiodothyronine concentrations of laying hens in both thermoneutral and heat stress and oestrogen (E_2) treatment groups. TN= thermoneutral; Veh= vehicle (control); HS= heat stress (means + s.e.m.; $n=6$).

4.2.2. Results of Experiment 2

In this study must be considered that the birds used herein were approaching the end of lay stage (80 weeks old). This might be contributable in resulting slightly different results compared to the results of **Experiment 1** and other results in the previous studies. Besides, the design of the experiment was also different, *i.e.* this study applied all treatments in only a controlled climate chamber. This means that the adaptation environment (22°C with 35% RH for two weeks) and followed by heat stress conditions (35°C with 55% RH) for two weeks, included oestrogen treatment in the second week of heat stress treatment, was carried out in the same climate chamber.

The results of the study in this trial again confirmed that heat stress caused an increased body temperature and reduced food intake as shown in **Tables 4.5**.

Table 4.5. Effects of heat stress and oestrogen on body temperature and food intake (means \pm s.e.m.; $n=7$).

		Adaptation	Heat stress	HS + E ₂
Body temperature (°C)	Control	40.9 \pm 0.04	41.9 \pm 0.08	42.1 \pm 0.07
	Treatment	40.8 \pm 0.05	42.0 \pm 0.07	42.2 \pm 0.09
Food intake (g)	Control	123.7 \pm 12.9	93.2 \pm 11.6	89.2 \pm 17.4
	Treatment	131.1 \pm 9.2	89.7 \pm 13.3	93.7 \pm 19.4

The changes of egg production before, during heat stress treatment and during both treatments of heat stress and oestrogen are shown in **Table 4.6**. Heat stress decreased egg number by 15% and 21% in control and treated group before the oestrogen treatment in week 1. During the week 2, when oestrogen treatment employed, heat stress reduced egg number by 70% in untreated group and only caused a 42% reduction in egg number in oestrogen treated hens. In this case oestrogen had been able to induce the heat stressed hens to produce a greater number of eggs than control (vehicle treated) hens.

Table 4.6. Weekly egg production changes before and during treatments ($n=7$).

		Adaptation	Heat stress	HS + E ₂	s.e.m.
Egg Number (n)	Control	34	29	12	11.5
	Treatment	33	26	19	7.0
\bar{x} yolk weight (g)	Control	18.7	18.5	18.8	0.15
	Treatment	18.8	18.6	18.9	0.15
\bar{x} egg weight (g)	Control	53.9	54.2	53.8	0.21
	Treatment	55.1	55.0	54.8	0.15
Σ yolk mass (g)	Control	637.1	536.8	225.1	214.8
	Treatment	619.8	482.6	358.4	130.7
Σ egg mass (g)	Control	1832.6	1570.9	645.6	623.6
	Treatment	1818.3	1430.0	1041.2	388.5

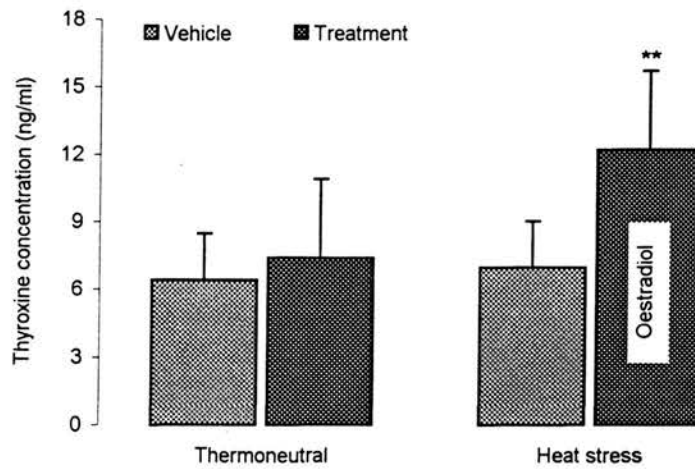
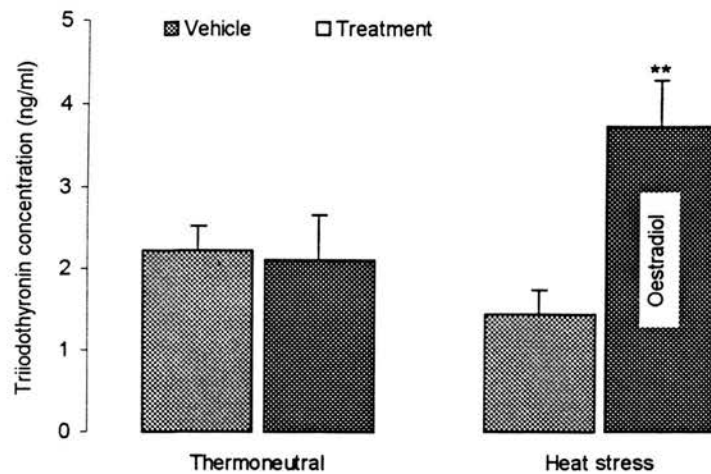
HS= heat stress, E₂= oestrogen , all treatments were applied in the same chamber.

Similar changes was also obtained in total egg mass production whereas control (untreated) hens lost over than 60% of the total egg mass, meanwhile oestrogen treated group had only a 43% reduction. Although oestrogen did not affect the yolk size and egg weight, it might still have an effect in improving egg production in heat stressed hens at the end of lay period.

Oestrogen did affect the egg yolk precursor concentrations in both thermoneutral and heat stress groups as can be seen in **Table 4.7**. Heat stress hens showed similar responses to the exogenous oestrogen administration in vitellogenin changes but did a different pattern within VLDL responses. It is likely that during exposure to high ambient temperature the birds did show a different responses to the oestrogen compared to those in thermoneutral environment. This conditions was entirely true for the VLDL changes rather than for the vitellogenin.

Table 4.7. Egg yolk precursor changes (means \pm SD; $n=7$).

		Adaptation	Heat stress	HS + E ₂
Vitellogenin ($\mu\text{g/ml}$)	Control	5.7 \pm 0.9	3.9 \pm 0.7	3.4 \pm 0.8
	Treatment	5.3 \pm 0.8	3.6 \pm 0.6	11.7 \pm 1.2
VLDL (mg/ml)	Control	16.7 \pm 2.9	12.3 \pm 3.6	7.2 \pm 3.4
	Treatment	17.1 \pm 3.2	10.7 \pm 3.3	25.7 \pm 4.4

**Figure 4.6.** Plasma thyroxine concentrations of laying hens in both thermoneutral and heat stress and oestrogen treatment groups (means + s.e.m.; $n=7$).**Figure 4.7.** Plasma triiodothyronine concentrations of laying hens in both thermoneutral and heat stress and oestrogen treatment groups (means + s.e.m.; $n=7$).

4.3. DISCUSSION

Results within this study again confirm that exposure the birds to high ambient temperature caused a heat stress conditions. The results in this study also confirm the effects of heat stress on egg production, egg yolk production and its precursor concentration changes as shown by the findings in the previous study (**Chapter 3.**). Heat stress decreased the availability of egg yolk precursors in the circulation which might be affected by the precursor synthesis in the liver. If this was the case -and since egg yolk precursor synthesis is strictly under oestrogenic control that transported in the plasma to the ovary (Wiskocil *et al.*, 1980; Bacon, 1986; Griffin *et al.*, 1984)-, the possible mechanisms was either by reduction in oestradiol secretion or reduced sensitivity of the hepatocytes to oestrogen stimulation.

Decreased precursor synthesis would account for the apparent reduction in plasma concentrations of vitellogenin and VLDL (Senior, 1974). The reduction in precursor availability may then result in decreased uptake by the oocyte, slower yolk accumulation and oocyte development and consequent changes in egg production. The changes in egg production, yolk size, egg yolk precursor concentrations and thyroid hormones in **Experiment 1**, however, were not similar compared with those in the **Experiment 2**. These included not only on the effects of heat stress on those parameters observed, but also on the effects and responses to oestrogen stimulation.

This findings could suggest that there was a different response to both heat stress and oestrogen stimulation between birds at peak of lay and those at the end of lay. For egg production hens at the end of lay period seemed to be easier affected by environmental changes than hens at the peak of lay in this study. They also showed different responses to oestrogen stimulation. Birds at the end of lay did not respond adequately ($P < 0.05$) to the oestrogen stimulation in both egg production and yolk size. Although, there was a significant different between oestrogen treated group and control hens at peak of lay. Oestrogen did not improve egg production in heat stressed hens at the end of lay period ($P > 0.05$), but did affect tremendously in birds at peak of lay ($P < 0.01$). Both groups still produced a remarkable improvement

compared to their appropriate control ($P < 0.05$), which shows that heat stressed hens are still capable of increasing egg production and egg yolk precursor in the circulation levels. This may assume that there was induction by oestrogen in the synthesis of egg yolk precursor in the liver.

Different responses between different ages of birds not only occurs in the egg production and egg yolk precursor concentration only, but also found in the thyroid hormone changes, both thyroxine (T4) and triiodothyronine (T3). Birds at peak of lay did not show any changes in thyroxine concentration but increased plasma triiodothyronine concentration. Meanwhile birds at the end of lay showed responses to oestrogen stimulation by increasing their plasma triiodothyronine concentration.

These findings in this study of heat stress reduced plasma T3 concentration agrees with Bobek *et al.*, (1980), Iqbal *et al.*, (1987) and Mitchell and Goddard (1990), but disagree with the findings that heat stress increases plasma T3 concentration (Cogburn and Harison, 1980). The different responses in the levels of T4 and T3 in heat stressed birds may also be affected by different humidity and chickens are able to divide high ambient temperatures into moderate and severe heat stress conditions (Kan, 1994).

Ovulation in a laying hen is maintained by regular increases in the plasma concentration of LH, progesterone, testosterone and oestradiol-17 β (Etches and Cunningham, 1977; William and Sharp, 1988). Exogenous LH can also stimulate the granulosa cells to enhance the production of progesterone (Culbert *et al.*, 1980; Hammond *et al.*, 1981) and stimulates production of oestradiol and andostenedione in theca cells (Marrone and Hertelendy, 1985). Having responses from heat stressed birds to the oestrogen stimulation in this studies may lead to a conclusion that heat stressed birds are still capable of synthesising egg yolk precursors and hence may maintain their egg production during exposure to the high ambient temperature when exogenous oestrogen was given. Although adaptive responses in the oocyte uptake mechanisms may occur concurrently during heat stress but have yet to be characterised in this study.

Based on the result of this study , it can also be suggested that liver of the heat stressed laying hen is still capable of synthesising vitellogenin and VLDL but hepatocytes sensitivity to oestrogen is probably altered during heat stress with or without reduced oestrogen in the circulation. It can also be assumed that during the exposure to high ambient temperature there is a reduction in the concentration of oestrogen in the circulation, although this requires further investigation on the determination of oestrogen concentration in the circulation of heat stressed birds.

CHAPTER FIVE

THE INFLUENCE OF HEAT STRESS ON PLASMA YOLK PRECURSOR LEVELS: THE RELATIONSHIP TO DEPRESSED FOOD INTAKE

5.0. INTRODUCTION

One potent and accessible modifier in the strategy for preventing heat stress is food intake that can exploit biological methods of reducing metabolic heat production (MacLeod and Jewitt, 1984). This study consistently used the body temperature as one of heat stress indicators (Francis *et al.*, 1991).

Results of the previous trials in this study (**Chapters Three and Four**) clearly show that chronic heat stress in laying hens considerably reduces yolk size, egg size and total yolk production concomitant with reduced egg yolk precursors in the plasma. These studies also demonstrated that the food intake was decreased in the laying hens when they were exposed to chronic heat stress. Suppression of food intake to avoid hyperthermia during extended exposure to high environmental temperature is a recognised thermoregulatory strategy in the domestic fowl (Smith and Oliver, 1971).

The availability of protein and fatty acids for the yolk and albumen synthesis in producing an egg is vital in addition to enough calcium, phosphorus and carbonate. These minerals are necessary for the production of egg shell in the shell gland. Equally important is the availability of energy from food intake. Any alteration in the food intake could affect the egg production. It, therefore, is necessary to investigate the relations between heat-stressed birds and those have the same food intake as heat-stressed birds but reared under thermoneutral conditions. It is also necessary to determine if the pattern of the decline in egg yolk precursor levels in the plasma mirrors the reduction in food intake. It is considerably important to establish the extent to which food intake may limit performance at high environmental temperatures.

This **Chapter Five** describes the changes in yolk precursor levels in the plasma and egg production of laying hens exposed to chronic heat stress and in others with comparable reduced food intake. The objective of the first study in this **Chapter** was to determine the influence of food intake on the laying performance of heat-stressed hens in comparison with those pair-fed hens kept under thermoneutral conditions. In the second section of this study the effects of different degrees of food restriction (85, 70 and 55%) under thermoneutral conditions have also been investigated.

5.1. THE INFLUENCE OF DECREASED FOOD INTAKE ON EGG YOLK PRECURSORS AND EGG PRODUCTION IN HEAT-STRESSED LAYING HENS (Pair feeding experiment)

5.1.1. Experimental Procedures

This study examines the changes in egg production and plasma yolk precursors in heat-stressed hens and in the pair-fed hens kept under thermoneutral conditions.

Twenty-four laying hens, 44 weeks old-ISA Brown, were used in the experiment. The hens were genetically selected for high egg laying performance. They were wing-tagged, weighed, grouped and randomly assigned to three groups ($n=8$). The hens were individually caged in three modules in two climate chambers. The same light regime of 14L:10D was maintained throughout the experimental period. All birds were kept for two weeks during the adaptation period at 21°C with 50% RH. The heat-stressed group was kept in a climate chamber at 35°C with 50% RH for two weeks. The other two groups were maintained in the other climate chamber at 21°C throughout the experimental period. The control and heat-stressed groups were fed *ad libitum*. The pair-fed group feeding was limited to the same amounts of food equal to those consumed by the heat-stressed hens on the previous day and they were kept in the control climate chamber (21°C with 50% RH), as illustrated in the **Table 5.1**. Water was available *ad libitum* through drinking nipples for all groups.

Table 5.1. Grouping of hens ($n=8$) indicating room temperatures with humidities and mode of feeding in all groups.

Group	Environmental condition	Mode of feeding
Control (Ctrl)	21°C and 50±5% RH	<i>Ad libitum</i>
Pair-feeding (PF)	21°C and 50±5% RH	Fed with the same amount or weight of food as consumed by heat-stressed hens on the previous day
Heat stress (HS)	35°C and 50±5% RH	<i>Ad libitum</i>

Feeding was done every day and weighing on the following day at approximately the same time for two weeks following the adaptation period. The pair-feeding food was divided into equally spread meals (four per day) to avoid the hens consuming the whole amount immediately and having artificial fasting for the rest of the day. Individual food intake and egg production were recorded and their mean values calculated. The percentage decrease in egg production between chronic heat stress hens and those maintained at the ambient thermoneutral temperature with pair-feeding was calculated and compared to thermoneutral control hens.

Blood samples were taken during the period of adaptation, as the baseline before applying the treatments (week 0), in the middle (week 1) and the end (week 2) of pair feeding and heat stress periods. Heparinised blood samples (± 2 ml) were obtained from the brachial vein by venipuncture. The plasma was collected by centrifugation at 1500 *g* for 10 min and frozen at -20°C prior to assay for plasma levels of the two major egg yolk precursors, *i.e.* vitellogenin (zinc) and VLDL (triglyceride). At the end of each experiment, birds were killed by a lethal dose of pentobarbitone (Rhône-Mérieux, Southampton, UK) injected intravenously through the brachial vein.

5.1.2. Results

The results of two-week heat stress and pair feeding treatments are summarised in **Figures 5.1-5.3** and **Tables 5.2-5.5**.

Body temperature, body weight and food intake

Similar to the previous results (**Chapters Three and Four**) heat stress in this trial also increased body temperature and reduced food intake and therefore body weight (**Tables 5.2 - 5.4**). Heat stress caused a hyperthermia that was indicated by a significant increase in body temperature ($P < 0.01$). Neither body temperature nor body weight was significantly different during the 'adaptation' period (**Tables 5.2 and 5.3**).

Table 5.2. Body temperature during the adaptation period and during the period when chronic heat stress was imposed ($n = 8$).

Week	Body temperature (°C)			Pooled <i>s.e.m.</i>	<i>P</i>
	Control	Pair-fed	Heat Stress		
0	40.78	40.81	40.83	0.03	NS
1	40.84	40.87	41.74	0.04	<0.05
2	40.89	40.83	41.82	0.05	<0.05

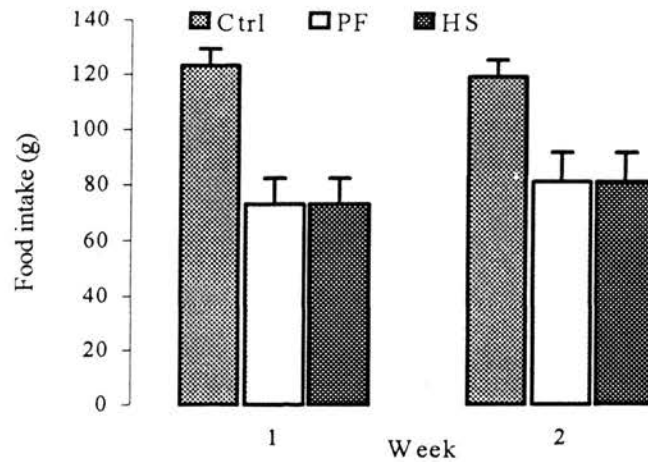
NS= not significantly different; week 0= thermoneutral and week 1, 2= heat stress conditions.

Restriction of food intake did not significantly decrease the egg production in pair-fed birds whereas it did in heat-stressed hens. The findings showed that better food conversion efficiency (FCE) in pair-fed hens (35% reduction). They could utilise food better compared to heat-stressed hens (24%) although they consumed the same amount of food. Earlier findings also found a similar response and suggested that decreased food intake was not the only explanation for decreased laying performance in heat-stressed birds (Smith and Oliver, 1972).

Table 5.3. Body weight changes during the adaptation period and during the period when chronic heat stress was imposed ($n=8$).

Week	Body weight (g)			Pooled <i>s.e.m.</i>	<i>P</i>
	Control	Pair-fed	Heat Stress		
0	2151	2196	2173	22.5	NS
1	2130	1891	2021	57.3	<0.05
2	2103	1756	1881	46.5	<0.01

NS= not significantly different ($P>0.05$); week 0= thermoneutral and week 1, 2= Heat stress conditions.

**Figure 5.1.** Means (+ s.e.m.) of food intake per bird ($n=8$) during heat stress and pair feeding treatments in all group during two weeks exposure to heat stress.

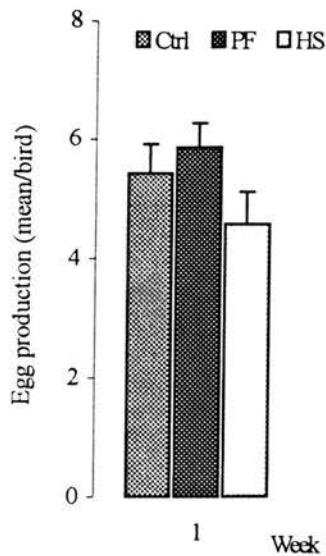
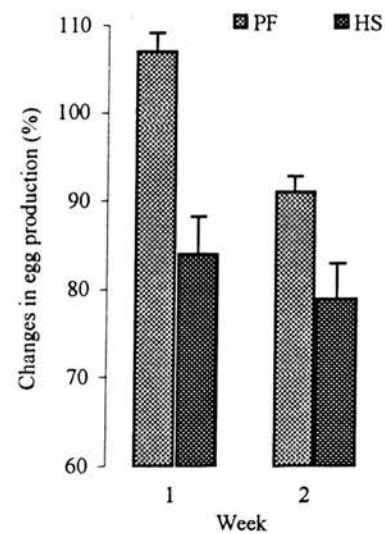
Egg production

When the treatments of heat stress and pair feeding imposed the body weight of pair-fed group was the lowest compared to those in the heat stress and control birds ($P<0.01$). This can be explained that the birds in pair-fed group still produced eggs whilst the heat-stressed birds although they consumed the same amount of food produced fewer eggs as shown in **Table 5.4.** and **Figures 5.2** and **5.3.**

Table 5.4. Egg and egg yolk production for control, pair-fed and heat-stressed groups during two weeks exposure to heat stress ($n = 8$).

		Control	Pair-fed	Heat Stress	Pooled s.e.m.
	<i>Week</i>				
Σ egg numbers (n)	1	35	36	32	2.10
	2	43	38	33	5.10
\bar{x} egg weight (g)	1	67.57	64.64	65.26	1.54
	2	68.09	63.85	64.32	2.32
\bar{x} yolk mass per egg (g)	1	17.45	16.90	17.33	0.29
	2	17.52	16.35	16.84	0.59
Σ yolk mass (g)	1	610.8	608.4	554.6	31.78
	2	753.4	621.3	555.7	100.70
Σ egg mass (g)	1	2365	2327	2088	150.16
	2	2928	2426	2123	406.58

Ctrl= Control; PF= Pair-fed; HS= Heat stress groups

**Figure 5.2.** Egg production produced by hen during heat stress and pair feeding treatments in all groups**Figure 5.3.** Changes (%) in egg production of treated groups in comparison to thermoneutral *ad libitum* group ($n=8$).

Plasma egg yolk precursors

Chronic exposure of the hens to heat stress caused a reduction in plasma egg yolk precursors as shown in **Table 5.5**. Heat-stressed hens exhibited reduced plasma levels of egg yolk precursor (both vitellogenin and VLDL) more severe than pair-feeding treatment birds although they consumed the same amount of food ($P<0.05$). The reductions of vitellogenin (as plasma zinc) and VLDL (as plasma triglyceride) were 9% and 33% in pair-fed birds. The decreases of vitellogenin and VLDL in heat-stressed hens were 25% and 38% respectively, although they were not different before the treatments.

Table 5.5. Plasma egg yolk precursor concentrations during the adaptation and the period when chronic heat stress was imposed (week 1 and 2; $n=8$).

Week	Zn ($\mu\text{g/ml}$)			Pooled		VLDL (mg/dl)			Pooled	
	Ctrl	PF	HS	<i>s.e.m.</i>	<i>P</i>	Ctrl	PF	HS	<i>s.e.m.</i>	<i>P</i>
0	4.29 100%	4.41 100%	4.64 100%	0.18	NS	12.04 100%	13.69 100%	11.78 100%	1.04	NS
1	5.16 +20%*	3.87 -12%	3.00 -35%	0.90	<0.01	14.83 +23%	8.48 -38%	6.49 -45%	3.36	<0.01
2	5.58 +30%	4.21 -5%	3.99 -14%	0.86	<0.05	15.16 +26%	9.82 -28%	8.18 -31%	3.65	<0.01

Ctrl= Control; PF= Pair-fed; HS= Heat stress groups; *) changes (%) in columns within group in comparison to the values in week 0..

In terms of food utilisation, pair-fed birds showed a better food conversion efficiency, *i.e.* the amount of food (kg) consumed for producing one kg of egg as shown in **Table 5.6**. Heat-stressed birds reduced their food efficiency in comparison with thermoneutral pair-fed hens ($P<0.05$).

Table 5.6 Food conversion ratio (FCR) changes in all groups throughout the experimental period ($n=8$).

Week	Food conversion ratio (food intake/egg production in g/g)		
	Ctrl	PF	HS
1	3.24 100%	1.84 ^b 57%*	2.10 ^a 65%
2	2.48 100%	1.78 ^b 72%	2.13 ^a 86%

Different superscripts in rows denote significant differences ($P<0.05$).

* Percentage changes in FCR compared to the appropriate control.

5.2. CHANGES IN PLASMA YOLK PRECURSORS IN LAYING HENS EXPOSED TO SOME DEGREES OF FOOD RESTRICTION

(Food restriction experiment without heat stress treatment)

The study on pair feeding in the previous section showed that during heat stress the birds reduced their food intake up to 25%. However, the identical reduction of food intake in the pair-fed birds indicated that the laying performance and circulating egg yolk precursor concentrations were higher compared with those of heat-stressed birds. It, therefore, is important to study more precisely the effects of food restriction *per se* on all parameters looking for relationships in laying hens kept under thermoneutral environment. This study also aimed to determine the extent to which restricted food intake may limit performance of laying.

In this study some different degrees of food restriction (85-, 70-, and 55%) were employed in comparison with *ad libitum* (100%) group under the same thermoneutral environment. This experiment compared the patterns of changes in the plasma yolk precursors (vitellogenin and triglyceride rich-VLDL) concentrations under different schemes of food restriction. The results were then compared with the response patterns of heat-stressed birds from the previous studies. In this study the level of food restriction was the only variable treatment. Other conditions were identical to the thermoneutral environment.

5.2.1. Experimental Procedures

Experimental birds

Twenty-four ISA Brown laying hens, 22 weeks old with high egg laying performance were used in this experiment. The hens were randomly divided into four groups ($n=6$). They were individually caged in two modules and kept under a controlled climate chamber at 21°C with a 24 h artificial lighting pattern (14L:10D photoperiod). To avoid tier effects, the birds of all groups were equally spread in all tiers (**Figure 5.4**). They were maintained for two weeks adaptation with access to food and water *ad libitum*.

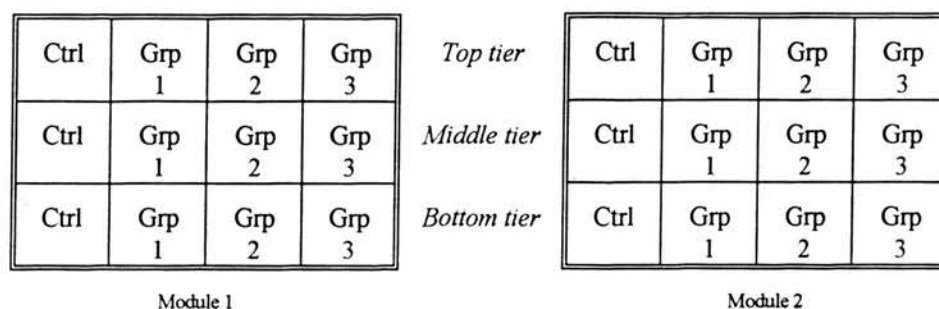


Figure 5.4. Arrangement of the birds in two battery modules kept in a controlled chamber.

Feeding

The restricting feeding was started following the adaptation period with hens in the control group received *ad libitum* (100%). Feeding of 85-, 70- and 55% food restriction were applied to the groups 1, 2 and 3 respectively. The restricted groups received the amounts of 85-, 70- and 55% of food consumed by control group on the previous day as summarised in **Table 5.7**. Feeding to the restricted groups was done every day and weighing on the following day at approximately the same time for two weeks observation following the adaptation week. The food for the restricted birds was spread into four feeding times to avoid the hens finishing the whole meal at one time and having artificial fasting for the rest of the day. The gap in the feeding times for the 70- and 55% restricted groups in this experiment however inevitably caused an artificial-temporary fasting. The egg production was recorded daily whilst body weight and body temperature were measured once a week before and during the restriction period.

Table 5.7. Grouping of hens ($n=8$) indicating room environment and mode of feeding.

Group	Mode of feeding	Environmental condition
Control	<i>Ad libitum</i> (100%)	[All groups were kept under the same environmental condition at 21°C and 50±5% RH].
Group 1	Restricted feeding 1 (85%)	
Group 2	Restricted feeding 2 (70%)	
Group 3	Restricted feeding 3 (55%)	

Blood samplings

Blood samples were taken during the period of adaptation, as the baseline before applying the treatments and in the early, middle and the end of restriction feeding period. Blood samples (\pm 2.0-2.5 ml) were withdrawn into heparinised tubes then centrifuged at 1500 *g* for 10-min to obtain the plasma fraction. Plasma obtained was then frozen at -20°C prior to assay for egg yolk precursors vitellogenin (zinc), VLDL (triglyceride), total calcium and macroprotein levels in the plasma. At the end of the study birds were killed by a lethal dose of pentobarbiturate (see **Chapter Two**: General Materials and Methods for details).

5.2.2. Results

Unlike the previous section, this study did not employ any heat stress treatment to the birds and the only treatment applied was different degrees of depressed food intake under thermoneutral conditions. The consequences of food restriction were then related to the yolk and egg production, and the circulating egg yolk precursors, vitellogenin and VLDL, in the plasma. The results were then discussed in comparison with the findings in the previous studies.

Food intake and body weight

The study employed 85, 70 and 55% food restriction in 1, 2 and 3 Groups respectively in comparison with the control group which was fed *ad libitum* (100%) in thermoneutral (22°C) conditions (**Figure 5.5**). Suppression of food intake in birds kept under normal ambient temperature did affect not only egg production but also reduced body weights. The reduction in the body weights was parallel with the degree of food restriction employed. **Figure 5.6** showed clearly this phenomenon where the severe decrease in body weight was Group 3 and the least was Group 1. The greater food restriction applied the lower energy intake the birds had. This resulted in the slower body weight gain as the birds were at early laying stage and might still grow.

The experimental birds in all groups consumed all the amount of food provided since the food was less than required. It, however, was difficult to avoid Group 3 had an 'artificial fasting' since this group had only 55% of the total food consumed by the control group. During three weeks experimental periods there was a small increase of food intake in control group and other groups consequently.

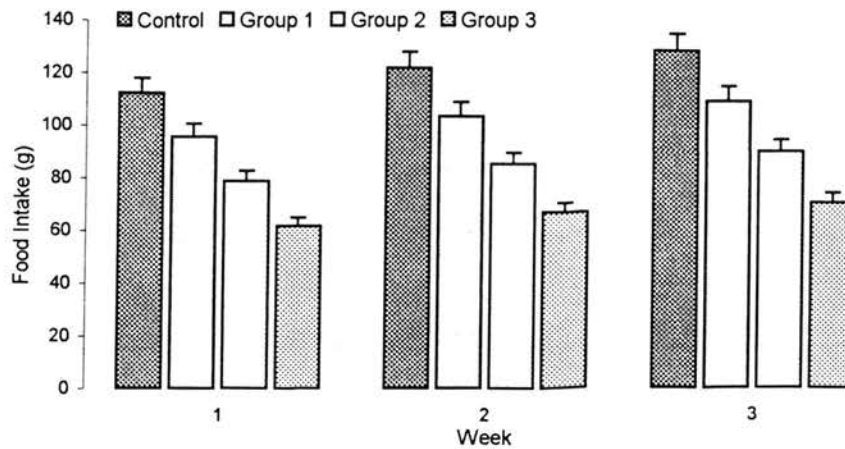


Figure 5.5. Food intake patterns of the restricted (85, 70 and 55%) groups in comparison with control (100%) group during the whole experimental periods.

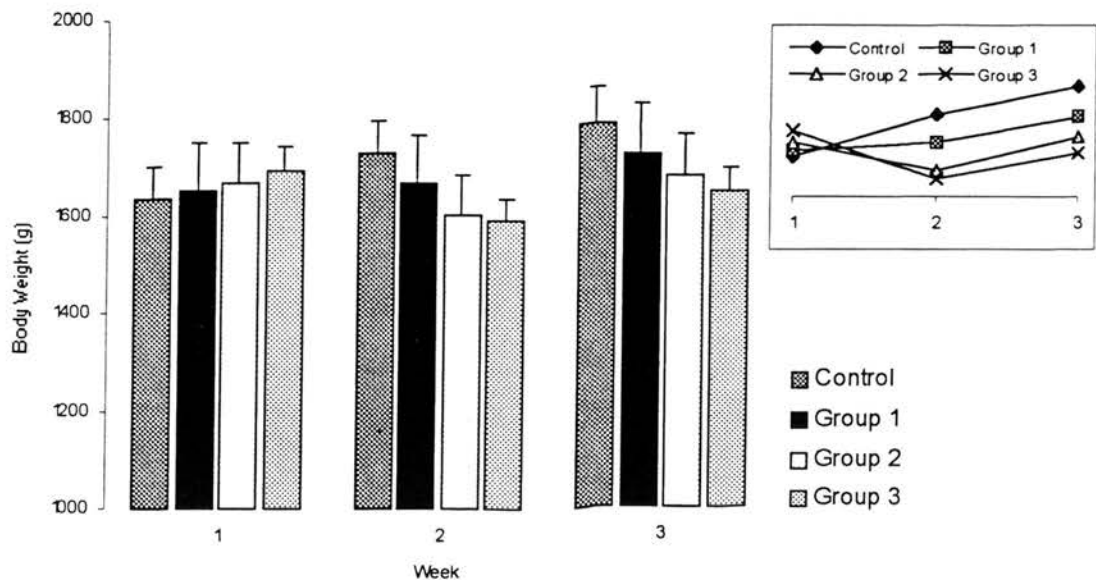


Figure 5.6. Body weight changes in restricted (85, 70 and 55%) groups in comparison with control (100%) group throughout the experimental periods. Inset box shows the body weight gain of the growing experimental birds.

Yolk and egg production

The results showed that food restriction affected both yolk and egg production probably, at least in a part, as consequence of the birds reduced energy intake. The pattern of food restriction was reflected in the reduction of egg production (**Table 5.8**). Group 3 as the most depressed in food intake (55%) showed the biggest reduction in both yolk and egg production. However, the changes in the egg production in week 2 were not similar with those values in week 3 in all restricted groups. Those changes were more clearly observed in term of total egg mass and egg yolk produced in weeks 2 and 3. The result of the study also showed that egg weight was the parameter which least affected by depressed food intake under thermoneutral condition.

Table 5.8. Egg production of restricted feeding groups in comparison with control group.

		Control	Group 1*)	Group 2	Group 3	Pooled s.e.m.
Egg Number (n)	Wk 1	40	41	39	40	0.82
	2	41	40	38	38	1.50
	3	43	36	29	21	9.43
\bar{x} yolk weight (g)	1	12.5	11.9	12.0	12.3	0.28
	2	13.5	13.0	12.7	13.1	0.33
	3	13.6	13.6	13.0	13.1	0.32
\bar{x} egg weight (g)	1	53.5	52.8	53.3	57.9	2.37
	2	58.0	55.1	57.2	54.6	1.63
	3	57.4	56.1	57.4	53.3	1.93
Σ yolk mass (g)	1	500.0	487.9	468.0	492.0	13.61
	2	553.5	520.0	482.6	497.8	30.79
	3	584.8	489.6	377.0	275.1	134.55
Σ egg mass (g)	1	2140.0	2164.8	2078.7	2316.0	100.80
	2	2378.0	2204.0	2173.6	2074.8	126.28
	3	2468.2	2019.6	1664.6	1119.3	370.12

*) Group 1,2 and 3 were 85, 70 and 55% food restriction groups respectively

The changes in egg production in this food restriction study were not similar (less severe) compared with those in heat-stressed hens with 25-30% food reduction in the previous studies (**Chapters Three and Four**). The results of this study showed that food restriction had no effects on egg production in the first week of treatment. Hens with up

to 30% food restriction (Groups 1 and 2) had a better egg production compared to those in heat-stressed hens in the previous studies with similar food reduction.

In addition the food restriction treatment up to 55% did affect egg weight but not yolk size throughout the experimental periods since Group 3 had bigger eggs initially as shown in **Figure 5.7** below. In the previous studies (**Chapters Three and Four**) heat-stressed hens which voluntarily reduced food intake showed decreases in egg weight and yolk size (around 8% reduction) and approximately a 35% reduction in 'total' weekly egg mass.

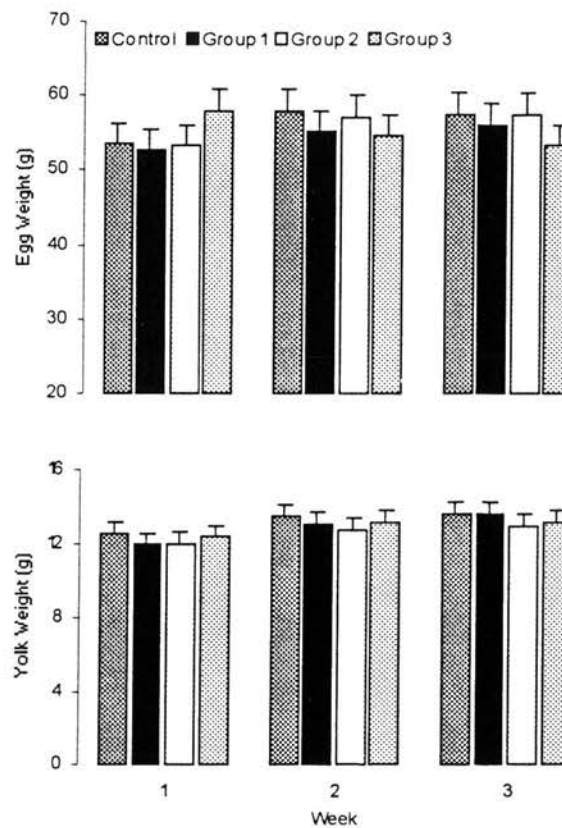


Figure 5.7. Egg and yolk weight produced by hens in restricted (85, 70 and 55%) groups in comparison with control (100%) group throughout the experimental periods.

Egg yolk precursors

Two major egg yolk precursors, *i.e.* VLDL and vitellogenin, were measured as plasma triglyceride and zinc concentrations respectively. The changes of these precursors

from the week of adaptation and to the weeks of food restriction for all groups were presented in **Figure 5.8**.

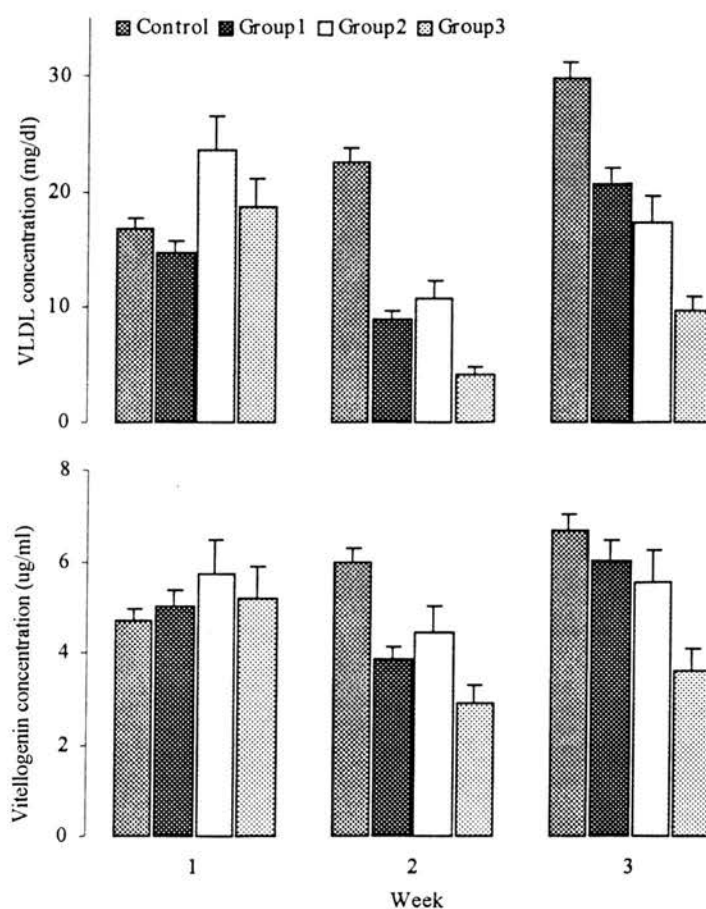


Figure 5.8. Changes in plasma yolk precursor concentrations in restricted (85, 70 and 55%) groups in comparison with control (100%) group. Plasma VLDL was measured as triglyceride and vitellogenin as zinc concentrations.

Since the birds used in this experiment were still in the early stage of egg production, the control group showed a constant increase in the VLDL as well as in its vitellogenin concentrations. The restricted groups dropped their concentrations within week 2 and increased in week 3. The restricted groups relatively decreased their concentrations in comparison with control group in week 3 although they were not severe than those changes in week 2. Those changes were clearly seen when the relative percentage of changes were plotted (**Figure 5.9**).

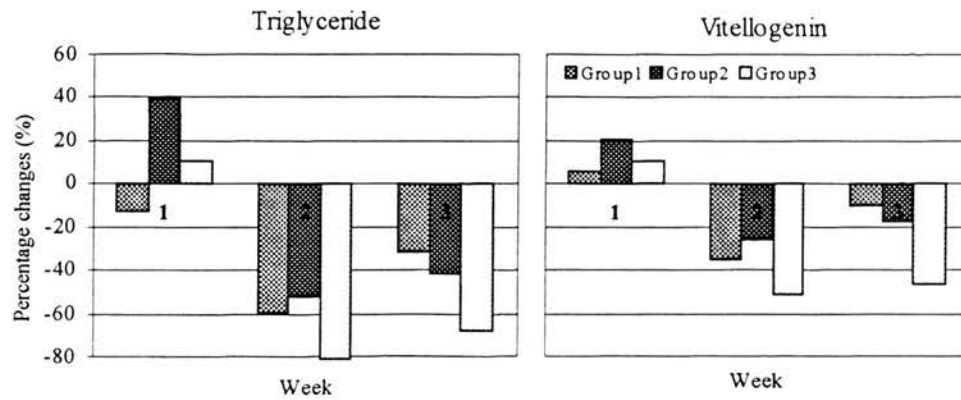


Figure 5.9. Percentage changes in plasma yolk precursor concentrations in restricted (85, 70 and 55%) groups in comparison with control (100%) group.

In addition, food restriction also decreased plasma concentrations of total calcium but had no effects on plasma glucose and protein (**Table 5.9**).

Table 5.9. Concentration changes in other plasma metabolites in the restricted (85, 70 and 55%) groups in comparison with control (100%) group.

		Control	Group 1 ^a	Group 2	Group 3		Anova (<i>P</i>)
Protein (mg/ml)							
Week	1	64.1	68.6	70.3	69.7	Week	0.010
	2	73.9	68.1	65.3	60.9	Food	0.450
	3	72.5	76.1	73.8	70.9	Week*Food	0.096
Glucose (mg/dl)							
Week	1	255.4	259.5	244.2	253.5	Week	0.189
	2	242.4	249.0	259.4	255.8	Food	0.994
	3	247.9	240.7	240.3	239.7	Week*Food	0.675
Total calcium (mg/dl)							
Week	1	5.83	6.19	7.38	6.37	Week	0.177
	2	6.67	6.17	5.67	2.66	Food	0.005
	3	8.09	5.89	5.41	3.87	Week*Food	0.065

^a Group 1, 2 and 3 were 85, 70 and 55% food restriction groups respectively

5.3. DISCUSSION

5.3.1. Pair Feeding Experiment

Suppression of food intake to avoid hyperthermia during extended exposure to high environmental temperature is a recognised thermoregulatory strategy in the domestic fowl (Smith and Oliver, 1971). Studies on the effects of thermal stress in the domestic fowls always show that heat stress decreases food intake (Sykes, 1979; Fisher and MacLeod (1985). Decreased food intake in heat stressed fowl is an adaptation of energy utilisation (Davis *et al.*, 1972; Van Kampen, 1974), which is also followed by some biochemical changes associated with changes in heat production (Mitchell and MacLeod, 1983). Avian species do appear to regulate food intake, and there is good evidence that both the gastrointestinal tract and the liver are primary sites for regulation (Denbow, 1995). Decreased food intake is also observed in pullets chronically exposed to high ambient temperature (Smith and Oliver, 1972).

Besides the reduction in voluntary food intake, thermal stress altered not only the behaviour of heat-stressed birds but also their production performance. It is likely that the reduction in laying performance observed due to heat stress are the result of complex physiological mechanisms. The physiological responses to heat stress involve the functional integration of several organs to meet the metabolic needs of birds that are trying to dissipate excesses heat. This may involve changes in hormonal balance and functions and the regulation of the blood flow (Bottje and Harrison, 1984). This study is aimed to examine the hypothesis that decreased food intake *per se* is not the only main factor in decreased egg production in heat-stressed hens. This objective can be achieved by applying a pair feeding experiment, *i.e.* by feeding pair-fed hens under thermoneutral environment with the same amount of food consumed by heat-stressed hens in the previous day. The results, especially in terms of egg production and plasma egg yolk precursor concentrations, then compared across all groups of control (*ad libitum*, thermoneutral), heat stress (*ad libitum*, high ambient temperature) and pair-feeding (pair-fed hens, thermoneutral).

Egg production

The results of this study showed that exposure of hens to high ambient temperature over a two week period causes heat stress conditions and detrimental effects on egg production. The results showed that heat stress caused a substantial decrease in egg production (**Table 5.4**). Heat stress also decreased yolk size and total yolk production. These decrease might be related to the synthesis and/or levels of the egg yolk precursors in the circulation

In terms of egg production, as similar to the findings in the previous studies, heat stress reduced egg production. The changes were concomitant with the decreases in the egg yolk precursor concentrations and food intake. The pair-fed birds showed no changes in the first week of pair feeding treatment but decreased their egg production by 10% in week 2. Meanwhile, heat-stressed birds showed 17 and 22% reductions in their egg production during the first and second week of heat stress exposure although they consumed the same amount of food. Again, the results of this study showed that pair-fed birds performed better than heat-stressed birds in terms of egg production although they consumed exactly the same amount of food. Similar findings also reported by Mitchell and MacLeod (1983) on their experiment upon Rhode Island Red laying hens. They found that the birds exposed to 32°C for 3 days exhibited a significant decrease in heat production concomitant with a marked decrease in food intake (23%). The decrease in food intake was significant after 24 h ($P < 0.05$).

It thus appears that exposure to heat stress reduces the number of eggs and thus the total yolk production. The appearance of reduced egg production in this study was concomitant with reduction in the availability of egg yolk precursors (vitellogenin and VLDL) in the circulation to which it may be attributable. However, pair-fed birds did not show a decrease in egg production as severe as heat stressed birds although consuming the same amount of food which means having the same amount of energy and nutrient intake.

Gilbert *et al.* (1983) suggested that the domestic fowl has two primary control mechanisms regulating ovarian function, *i.e.* the regulation of the number of follicles initially starting growth and the regulation of the rate of atresia in the small follicles. Atresia might also occur to growing large follicles as proposed by Waddington *et al.* (1985). They found that under adverse conditions ovarian regulation has another way of

modifying the ovulation rate by eliminating the large follicles through atresia. This condition might be implied in the decrease of egg production during chronically exposure to heat stress.

In addition, the decrease of egg production in pair-fed hens was smaller compared with those values in heat-stressed hens which reduced the food intake by 25-30% in the previous studies (**Chapters Three and Four**). Pair feeding did not significantly affect egg weight and yolk size through out the experimental periods as shown in **Table 5.5**. Pair feeding, however, did have significant influence in the total egg mass and total yolk produced ($P<0.05$) in comparison to those in control hens. Heat-stressed hens had greater reduction in egg number, total yolk mass and total egg mass produced than both control ($P<0.01$) and pair-fed groups ($P<0.05$). In the previous studies (**Chapters Three and Four**) heat-stressed hens, which voluntarily reduced food intake by 26%, showed decreases in egg weight (16% reduction) and yolk size in the first week of treatment. Pair-fed hens showed better egg production in overall compared with those of heat-stressed hens in the previous studies.

Plasma egg yolk precursor concentrations

Results in **Table 5.3** showed that before heat stress treatment (week 0) there was no difference in both plasma zinc and VLDL concentrations between the two experimental groups compared to control groups. However, over the subsequent two weeks of heat stress and pair feeding treatments there were overall significant reductions in vitellogenin (plasma zinc) and VLDL (plasma triglyceride) concentrations in the heat-stressed group ($P<0.01$). Vitellogenin reduction was greater in heat-stressed hens (-35%) than pair-fed hens (-12%) in week 1 of heat stress treatment. Similarly, VLDL concentrations showed 45% and 37% reductions in heat-stressed and pair-fed groups. The plasma of laying hens contains high concentrations of triglyceride-rich lipoproteins. These are very similar to the yolk triglyceride-rich lipoproteins and this strongly suggests there is a direct transfer of lipoproteins from plasma to yolk (Bacon *et al.*, 1973; Christie and Moore, 1972). This may lead to an assumption that reduced precursors' availability in the circulation may also be responsible for the decreased egg production in heat stressed hens. The egg number

decreased by 17% and 5% in heat-stressed and pair-fed groups respectively or 21% and 11% reduction in terms of total egg mass produced.

The two major egg yolk precursors (vitellogenin and VLDL), which are synthesised by the liver, are the main factors required for egg yolk formation. The availability of these precursors in the plasma is vital for the growing oocytes. Any conditions causing a reduction in their availability may lead to disturbances in egg production. Decreased yolk precursor synthesis would account for the apparent reduction in plasma concentrations of vitellogenin and VLDL.

5.3.2. Food Restriction Experiment

In this study food restriction was applied to the experimental hens without any heat stress treatment. The study was aimed to determine whether the pattern of changes in egg yolk precursors and egg production under food restrictions was similar to those in heat-stressed hens with voluntarily decreased food intake. The changes in egg production and egg yolk precursor concentrations of restricted groups were then compared to control (*ad libitum*). The changes were also discussed and compared to those changes in heat-stressed hens in previous studies. It should be taken into account that the birds employed in this study were layers at early stage of laying whilst the birds in the pair feeding experiment were at about after the peak of lay (44 weeks of age) which may exhibit slightly different responses and affect the model.

Egg production requirements include both those linked to the product and that energy associated with synthesis process. The hens must meet their energy requirements that consist of those associated with egg production and those linked to tissue growth and energy expenditure (Romijn and Vreugdenhil, 1969; MacLeod *et al.*, 1993). Energy requirements correspond to egg production which change with the rate of lay, egg weight and its composition (Larbier and Leclercq, 1994). Although birds with body weight loss may have a better blood composition in terms of the number of red blood cells per unit volume as reported by Buhr and Cunningham (1994). They found that hens with lost 25% of their body weight had higher packed cell volume (36%) than hens that had body weight loss of 20% (34%) or hens that lost 15% (33%). The pattern of decrease egg production

in hen with decreased food intake in thermoneutral, however, is not similar as of heat stressed hen. However, reduced food intake does not necessarily mean that it would give similar effects on the changes of plasma yolk precursors and egg production in hens under thermoneutral compared to the heat-stressed hens.

The results of this study showed that under thermoneutral environment food restriction did apparently decrease egg production in the second week whilst the first week production was not affected at all. This condition led to an assumption that it took more than a week for the egg production to be influenced by the reduced energy intake through restricted food consumption. It seems that the fast growing oocytes in the hierarchy were not affected by the food restriction.

Egg weight and yolk weight were not significantly affected by food restriction treatment throughout the experimental period although there was a 6% difference. Food restriction to 55% of the normal food intake did not significantly affect the size of the egg and the yolk, at least in two weeks observation (**Figure 5.7**). In this regards, these effects were very similar in magnitude to egg weight response to heat stress although they had about 25% voluntary reduction in their food intake. Under thermoneutral conditions there may be a factor(s) controlling the production of egg to ovulate a certain size of yolk and produce particular size of egg. However, this study did not determine these particular aspects.

Food restriction treatment did affect the egg yolk precursors availability in the circulation. Regardless of the effect of food restriction on egg production during the first week, both two major egg yolk precursors, triglyceride rich-VLDL and vitellogenin, were decreased during the first week of the treatment (**Figure 5.7**). The percentage changes of these precursors were greater in the triglyceride rich-VLDL than vitellogenin but those values showed a similar pattern (**Figure 5.8**).

The birds employed in this experimental were just about at point of lay (22 weeks old) and they naturally gained their egg production to reach the peak level of laying performance and still growing. Larbier and Leclercq (1994) suggested that the pullet at point of lay has not yet completed its growth. The bird continues to develop for several weeks, independently of its fat depots. Therefore, the hens in the control group showed a constant increase in their yolk precursor concentrations. However, the remaining treated

groups failed to reach the level of the control group. There was no differences between group 2 and 3 (85 and 70% food restriction) in their precursor concentrations. Similar with the results in the egg production, the decrease of egg yolk precursor in the plasma concentrations of treated groups were parallel with the degree of the food restriction. The changes in the concentrations of egg yolk precursor of the birds kept under thermoneutral environment related to the decreased food intake whereas from the results of the previous studies showed that changes in egg yolk precursor concentrations did not correlate to the changes of food intake.

Food restriction had an effect on the plasma concentration of total calcium but did not affect the glucose and protein concentrations. There was no interaction effects of week and food restriction for these metabolites concentration in the plasma. Again in these results of this study showed that the decrease of food intake in the hens kept under thermoneutral were not similar with those values in the heat-stressed hens. From these results it can be concluded that reduced food intake is not the only main cause in the decreased egg production. Presumably the hens had food restriction but kept under thermoneutral condition might have a better food utilisation in some extents than those suffering under heat stress. As some workers demonstrated that hens consume less food during exposure to high ambient temperature, heat stress reduces egg production egg size and egg shell thickness (De-Andrade *et al.*, 1977; Miller and Sunde, 1975; Deaton, Reece and Lott, 1981; Deaton *et al.*, 1982; Yamada and Tanaka, 1988).

In comparison to the results of the previous sections (pair feeding trial and studies in **Chapters Three and Four**), food restrictions showed different effects on egg yolk precursor and egg production changes. Patterns of those changes in heat-stressed hens were not similar with those in thermoneutral hens. This study support the earlier results (pair feeding study) that the changes in egg yolk, egg production and plasma yolk precursors during heat stress are not mainly caused by decreased food intake. Although decreased food intake is one of contributable factors which responsible in decreased egg production in heat-stressed hens.

It has been suggested that yellow follicles are recruited from the pool of small white follicles in the ovary by a continuous growth process, as happens in the domestic fowl (Gilbert *et al.*, 1983). The dynamics of ovarian follicular populations are therefore of

fundamental importance in determining rates of lay (Hocking *et al.*, 1991). In this regards heat stress may be as a considerable factor causes in disturbances of the follicular development, this includes altering the deposition of yolk precursors into the growing oocytes.

The findings in this study showed that the reduction in food intake did not show a similar pattern with the decrease in egg yolk, egg production and plasma concentrations of egg yolk precursors. The reduction in food intake also caused different responses between heat-stressed hens and those of hens in thermoneutral environment.

From the results of pair feeding and restricted feeding studies above it could be concluded that the decrease in egg production which concomitant with decreased circulating egg yolk precursors in heat stressed hens is not mainly due to the decreased food intake. Reduced food intake is therefore can be suggested as one of the contributable factors in decreased egg production and plasma yolk precursors in heat-stressed hens.

The reduction in the availability of egg yolk precursors in the circulation may then result in decreased uptake by the oocyte, slower yolk accumulation and oocyte development and consequent changes in egg production. Adaptive responses in the oocyte uptake mechanisms may occur concurrently during heat stress but have yet to be characterised. It is, therefore, necessary to determine the uptake of egg yolk precursors in the ovarian level with an appropriate technique.

CHAPTER SIX

DETERMINATION OF THE EFFECTS OF CHRONIC HEAT STRESS UPON EGG YOLK PRECURSORS UPTAKE BY OOCYTES *IN VIVO*: INTRODUCING A NEW TECHNIQUE

6.1. INTRODUCTION

The physiological responses in heat stressed birds may be mediated by an altered liver synthesis of egg yolk precursors. The decrease might also be accompanied by a reduction in the uptake of precursors in the ovarian level that resulting in smaller and fewer yolk. Heat stress also induced decrease in total yolk mass output and this could be a consequence of a slower rate of precursors accumulation in the growing oocytes. However, the mechanism mediating this response awaits elucidation. The contributing factors may include inhibition of hepatic yolk precursor synthesis, perhaps due to altered oestrogen secretion, reduced availability of precursors in the circulation and decreased uptake of precursors in oocytes.

This **Chapter** describes further experiments carried out to determine whether the mechanisms mediating reduced egg production in heat stressed laying hens involve a change in the rate of uptake of yolk precursors by the oocytes. This has led to the establishment and validation of a new technique in allowing quantitative characterisation of uptake of yolk precursors by the oocytes *in vivo*. It is suggested that it may be possible to exploit the high specificity and capacity for binding of zinc exhibited by vitellogenin in order to achieve this objective.

Based on the ability of the vitellogenin's property to bind zinc, a sample of plasma was radiolabelled with radioactive zinc (^{65}Zn ; as ZnCl_2). The radiolabelled vitellogenin was separated from other plasma components using chromatography by gel filtration which was then concentrated by centrifugal ultrafiltration. The purified vitellogenin was then injected into the laying hens and accumulation of the labelled

vitellogenin by oocytes and liver measured in order to quantify the rate of uptake. When the technique was developed, it was validated by repeating it on two laying hens (control and heat stress) in order to determine whether the mechanism mediating reduced egg production in heat stressed birds involved a change in the uptake rate of yolk precursors by the oocytes.

The development and validation of this latter technique and its concurrent application with measurement of circulating yolk precursor concentrations is proposed as an important and useful method for progress in elucidation of the mechanisms involved in the possible inhibitory effects of chronic heat stress upon oocyte yolk accumulation and egg production. This study was an introductory in establishing a new technique which meant there was no specific or particular reference available. These included the absence information on the quantity of the radioactive zinc (^{65}Zn) must be added to the bulked plasma, concentration and volume of radiolabelled vitellogenin to be injected to the hens. In addition, the appropriate setting up for the column (speed of the micropump, diameter of the micropipe and other volumetric aspects) required such technically validations. Therefore, some results from other trials within this study were necessarily included in this section in order to provide further explanations.

The aim of this study therefore was to extend the knowledge of the effects of chronic heat stress on egg yolk precursors uptake in laying hens; the main objective was to develop and validate a technique allowing quantitative characterisation of the uptake of yolk precursors by oocytes *in vivo*. The second objective was to compare the uptake of yolk precursors by the oocytes in control and heat stressed hens using the technique.

6.2. MATERIALS AND METHODS

6.2.1. Experimental procedures

Fourteen laying hens, 52 weeks old, from a commercial line (ISA Poultry Services, Peterborough, UK) with high egg productivity were used in these experiments. All birds were kept in individual cages and received food and water *ad*

libitum. Birds were divided at random in two groups, one experimental and one control. Birds used in this experiment were all approximately in the same body weight (1990 ± 26.5 g). The birds from both groups ($n = 7$) were kept in controlled climate-chambers. The control group was kept under thermoneutral conditions (22°C with 50% relative humidity/RH) whereas the heat stress group were housed at $35^{\circ}\text{C}/60\%$ RH. All the birds received a lighting pattern of 14L:10D. At the end of the experiment, birds were killed by administration a lethal dose of Pentobarbitone sodium (Rhône-Mérieux, Southampton, UK) injected intravenously through the brachial vein. Eggs were collected and weighed as soon as they were laid between 10.00 to 18.00 h daily; all eggs collected during a day were assumed to be the egg production for that group on the day specified. Broken eggs were also included for egg production, as well as their yolk weights. After weighing the eggs were broken and the albumens were separated from the yolk and the means of each group were then worked out on daily basis.

All of the plasma metabolite assays (total zinc, VLDL depleted zinc, VLDL and macroprotein) had been modified and previously validated for use in avian systems and were performed by an automated plate reading spectrophotometer (Titer-tek 2, Autoflow Laboratories UK) as described in **Chapter Two: General Materials and Methods**.

The radiolabelled vitellogenin was injected to other hens to determine the distribution of the protein and to estimate the uptake rate by individual oocytes by injecting to other laying hens. The study then determined the optimum vitellogenin administration protocols, experimental duration and sampling techniques to provide the specific oocyte uptake data. This entailed determination of the distribution of the labelled ligand in a number of tissues and plasma and within the oocyte yolk.

6.2.2. Preparation for Vitellogenin Radiolabelling

To have a stock of ^{65}Zn labelled plasma, 3 regularly laying hens were blood sampled and the samples obtained were then mixed. By utilising the vitellogenin's property of Zn^{2+} binding, vitellogenin was isotopically labelled by incubation with

radioactive ^{65}Zn (as ZnCl_2). The amount of 200 μl of radioactive ^{65}Zn (1.85 MBq) was added to 4 ml of the 3 hens' bulked plasma. Details of the preparations and necessary records are described in **Appendix 1**.

Gel Filtration Chromatography

The chromatography by gel filtration technique is based on the size of molecules. The immobile phase was constituted by an Agarose gel, in the form of spherical beads, specially prepared for gel filtration in aqueous media. The exclusion limit was 5×10^6 molecular weight (MW); because the operating range was 10×10^3 to 5×10^6 MW, the column could separate any solution containing molecules within that range. The mobile phase was a buffer containing (a) NaCl, 20 mM Tris and (b) PMSF (50 mg/ml) which was adjusted to pH 7.4 with 20mM HCl

The gel was then mixed completely until no settled precipitate remained and some freshly made buffer was added and shaken. The solution was then poured into the column with the bottom tap open. The gel was allowed to settle but not to dry; buffer was added until the column was almost full. The gel was continuously added until there was a definite layer of gel at the top. Further buffer was then added to give a 2-3 mm layer on top. The bottom tap was closed after the drips had stopped. The pump was connected to the column and pumped the buffer through the gel; the buffer coming out was collected. The column dimensions were 45 cm high and 3 cm in width as illustrated in **Figure 6.1**. below.

Column Calibration

The column had to be calibrated several times in order to establish a standard flow rate. The column was run overnight using Bio-rad gel calibration standard. This standard contains 4 proteins (of which one is coloured) and two coloured markers (see **Table 6.1**). The coloured markers were helpful in detecting the total elution volume of the standard and useful to check whether the column running well with no air bubbles and was well seated. All the fractions were collected and a protein assay was done to determine their elution profile.

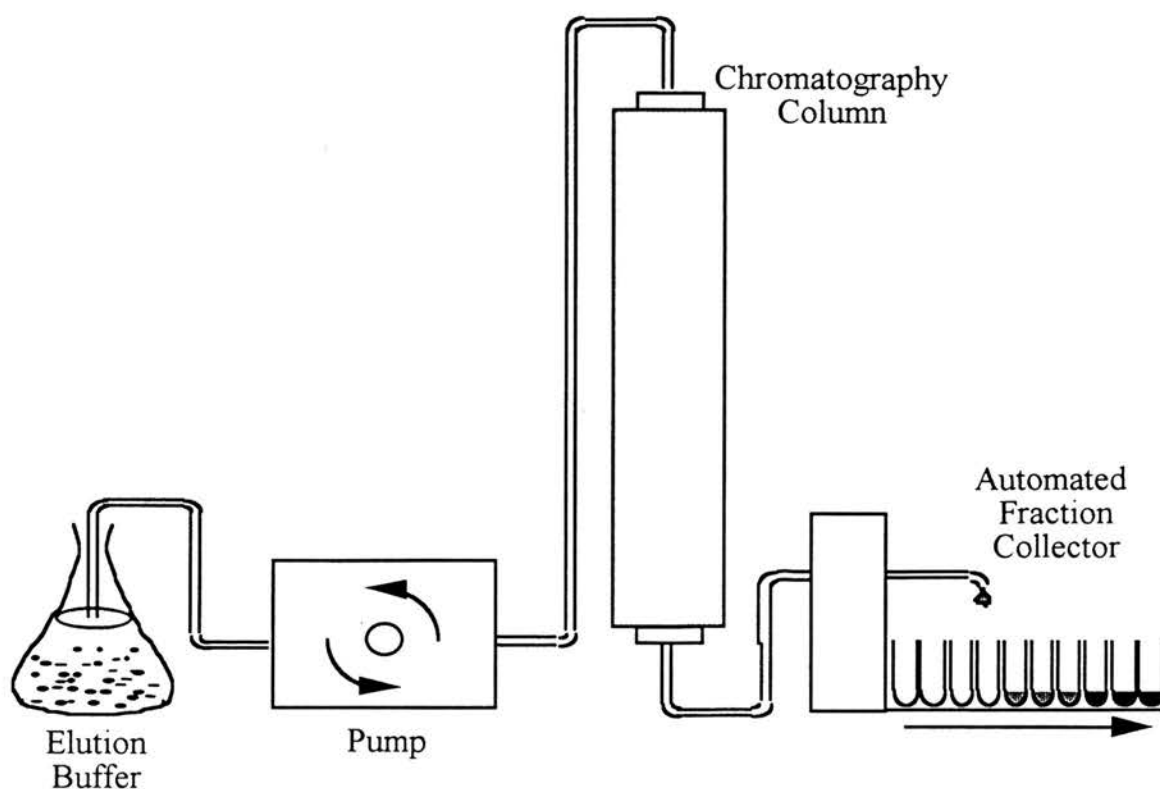


Figure 6.1. A schematic diagram of the chromatography column

Table 6.1. Composition of the chromatography standard solution

Markers	Quantity (for 2 ml buffer)	Molecular weight (Da)	Colours
Dextran blue	1 mg	2.00×10^6	Blue
Thyroglobulin	5 mg	0.67×10^6	-
Bovine γ -globulin	5 mg	0.16×10^6	-
Chicken ovalbumin	5 mg	44×10^3	-
Equine myoglobin	2.5 mg	17×10^3	Brown
Vitamin B ₁₂	0.5 mg	1350	Purple

Purification of the Vitellogenin

Based on its molecular weight and the protein calibration profile the elution volume of vitellogenin was established. Radiolabelled plasma was loaded into the column and the concentration was eluted. Because ^{65}Zn is a very high energy γ -

emitter, it was easily detected when run through the column and later in the collected fractions. Each fraction was counted using a Geiger- γ -counter (Mini Instrument Well Probe Type 43 SER, LKB-Pharmacia, UK) and protein, zinc and triglyceride assays were carried out to determine their elution profiles. Fractions containing identified vitellogenin were bulked to prepare them for the ultrafiltration.

The ultrafiltration technique is also based on the size of the molecules and produced a highly purified Radiolabelled vitellogenin sample. In each tube 2.5 ml of pooled purified vitellogenin were distributed and the filters (cut off at 100 kDa) settled down on the liquid. They were centrifuged at a gradually rising speed and time (5 min at 2000 rpm, followed by 10 min at 5000 rpm). Purified and concentrated vitellogenin was then collected in the bottom of each tube and bulked. An aliquot was taken, counted for radioactivity and analysed for protein and zinc. Purity of the labelled vitellogenin was assessed by precipitation of protein using an equal volume of an aqueous solution containing 0.02% dextran sulphate, 200 mM MgCl and 10mM Tris-HCl adjusted to pH 7.4 as previously described (Griffin and Mitchell, 1984). The supernatant was then assayed for protein zinc and radioactivity.

6.2.3. Radiolabelled Vitellogenin Injection

Before injection of vitellogenin the hens were weighed; blood samples were taken for zinc, protein and triglyceride assays to determine the plasma base line concentrations of these metabolites. The hens then was injected intravenously through the brachial vein with purified ^{65}Zn -labelled vitellogenin (3.5 ml). Following a period to allow equilibration of the isotope with the *in vivo* vitellogenin zinc pool; blood samples were taken at 2, 15, 30, 45, 60 and 75 mins after injection and collected into heparinised tubes. The previous trial showed that equilibration of the isotope with the *in vivo* pool occurred within 2 min and that the radioactivity was almost completely (95%) removed from the circulating blood within 90 min post injection. The birds were then killed and the oocytes and livers collected, weighed and counted for radioactivity.

6.2.4. Determination of Vitellogenin in the Fluid and Tissues

The whole blood, plasma, liver and oocytes obtained were counted on a γ -counter (LKB-Pharmacia, UK). The distribution of the labelled ligand was determined in a number of tissues (liver and muscle), plasma and within the oocyte yolk. Each tissue was weighed, homogenised and counted with the appropriate counting geometry. Oocytes were also weighed in order to calculate the uptake per gram tissue. The blood samples were assayed for the macroprotein, vitellogenin and VLDL concentrations. Protein assay results were believed to represent fractions containing low density lipoprotein, vitellogenin and albumen. These results were plotted together with the radioactivity counts and yielded identifiable elution profiles. The rate of uptake by the oocytes, distribution in the tissues and elimination rate of ^{65}Zn in the blood circulation could therefore be determined.

6.3. RESULTS

Some results from other trials within this study were necessarily included in this section in order to provide further detail explanations.

6.3.1. Egg production

The result of this study was also taken to confirm that heat stress reduces egg production and was important in considering ovarian uptakes. The data on egg production of each bird were analysed within and between groups and were compared on a weekly basis. During the early phase of the experimental period total weekly egg production was consistently lower (15%; $P<0.05$) in the heat stressed group as compared to the control birds. However, over the rest of the experimental period the decrease represented a bigger reduction (27%; $P<0.001$). The data are presented collectively as total weekly egg production and are shown in the **Figure 6.2**.

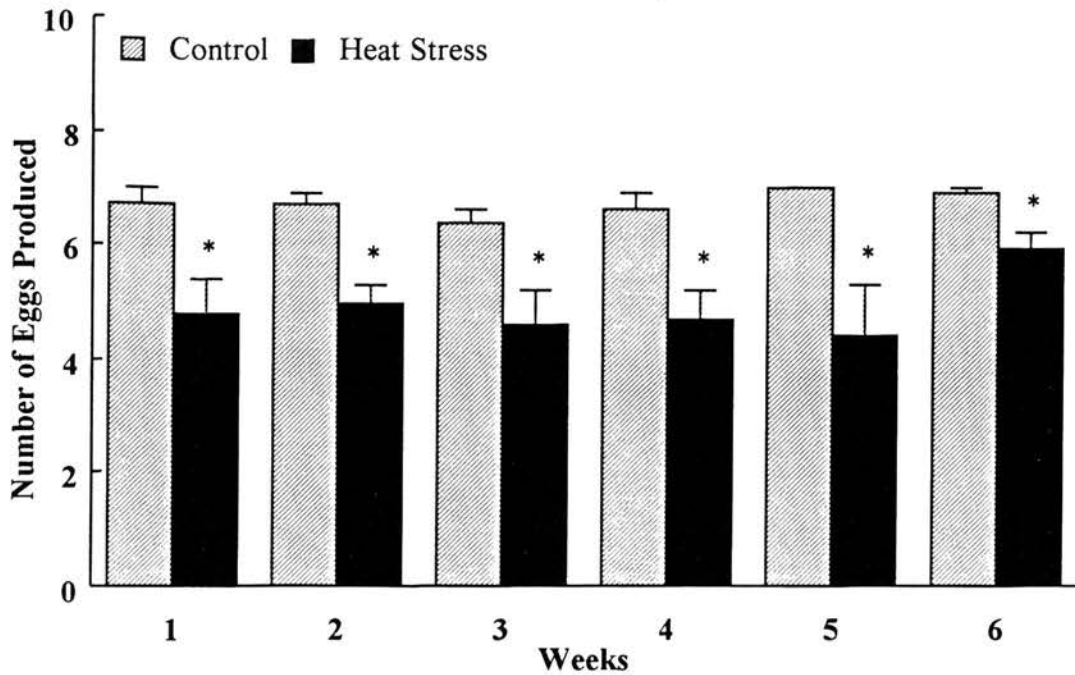


Figure 6.2. Total weekly egg production for control and chronically heat stressed birds ($n=12$). Values represent means \pm s.e.m. '*' significant difference ($P<0.05$).

The egg weight data are presented in terms of mean egg weight weekly (**Figure 6.3**) as well as per day and total egg weight throughout the experiment (**Figure 6.4**). The egg yolk data are presented in the same ways as shown in **Figure 6.5** and **6.6** which show a comparison of weekly yolk weight production in the two groups over the whole experimental period.

Heat stressed birds showed a very significantly lower ($P<0.001$) in their egg weight production in comparison with the control group. This decrease represented approximately a 40% reduction in total weekly egg mass in the heat stressed group. Similar responses were also found in the estimated mean of egg weight per day and weekly which were much lower (15%; $P<0.001$ and 37%; $P<0.001$ respectively) in the heat stressed birds compared to the control (**Figure 6.4**). The observed difference in the percentage reduction of egg weight measurements between the estimated mean egg weight per day and mean egg weight is due to the disparity in the number of eggs produced given that the heat stressed group laid fewer eggs.

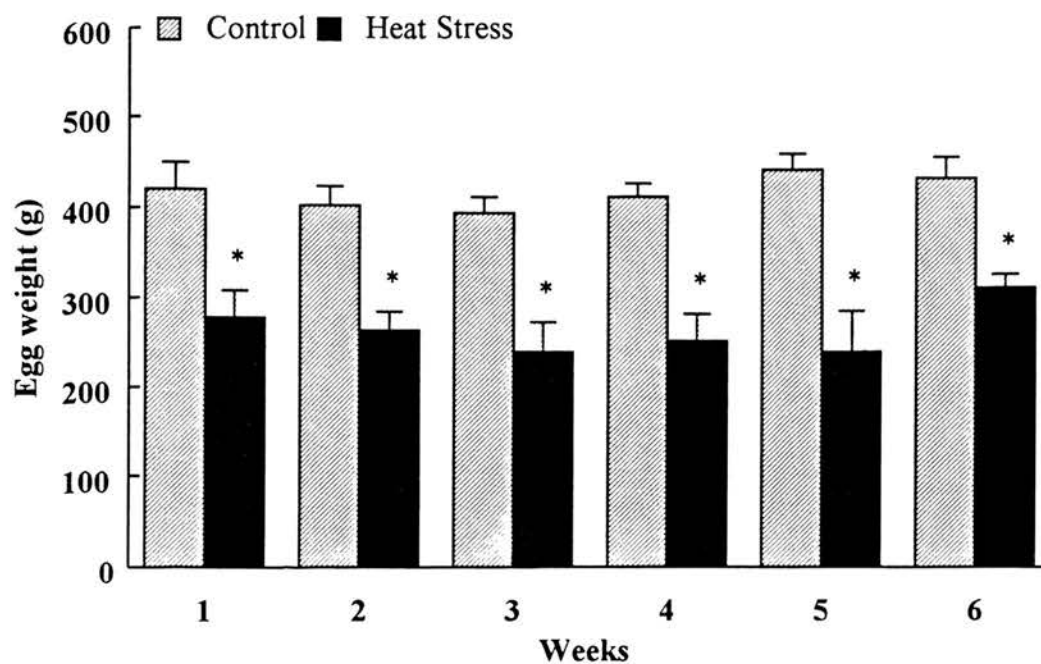


Figure 6.3. Total weekly egg weight production for control and chronically heat stressed birds. Values represent means \pm s.e.m.. '*' denotes significant difference ($P < 0.05$).

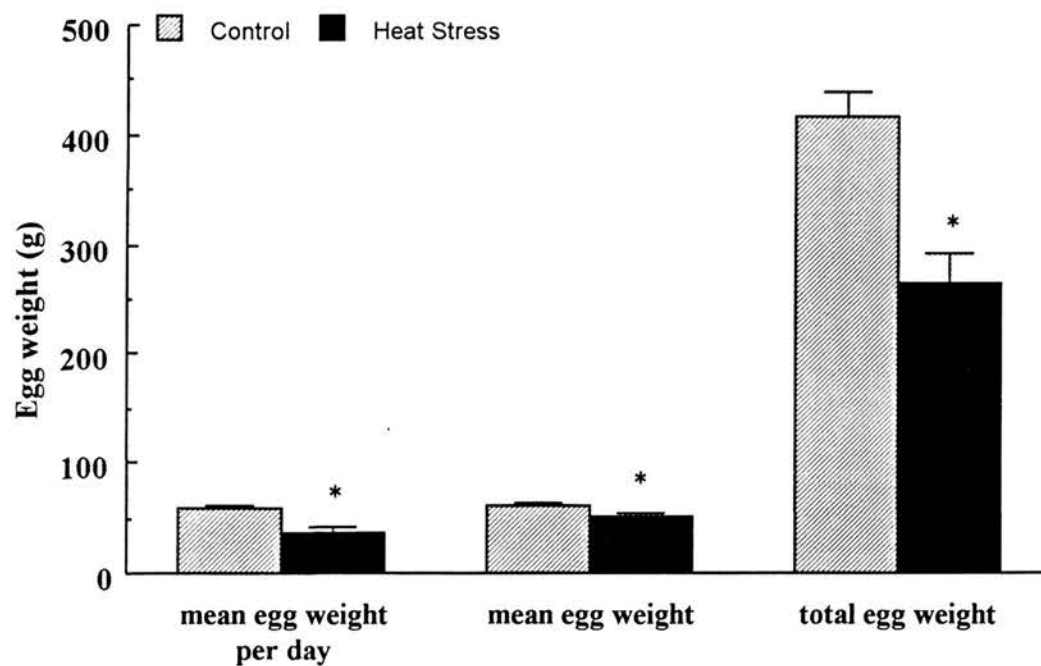


Figure 6.4. Effect of heat stress on mean egg weight for control and chronically heat stressed birds. Values represent means \pm s.e.m.. * denotes significant difference ($P < 0.05$).

Figure 6.5. shows that the total yolk weight of the control group was significantly higher ($P<0.001$) compared to the heat stressed birds. The decrease represented approximately a 35% reduction in total weekly yolk mass during the whole experimental period (Figure 6.6.).

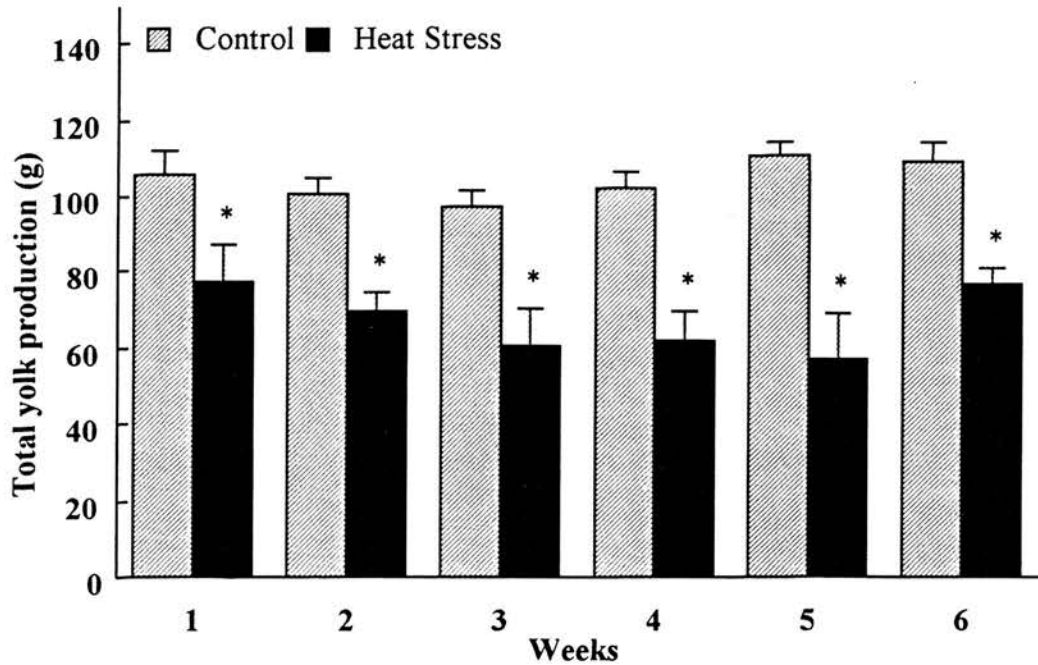


Figure 6.5. Effect of heat stress on total yolk weight for control and chronically heat stressed birds. Values represent means \pm s.e.m.
* denotes significant difference ($P<0.05$).

Estimated mean yolk weight per day and mean yolk weight for the control and heat stressed treatments are also presented. The means of the egg yolk weights were also lower in the heat stressed group (14%; $P<0.001$) as were the mean yolk weights per day (37%; $P<0.001$). Again the observed differences in the percentage reduction of egg yolk weight between the estimated mean yolk weight per day and mean yolk weight were due to the difference in the number of eggs produced between the two treatment groups.

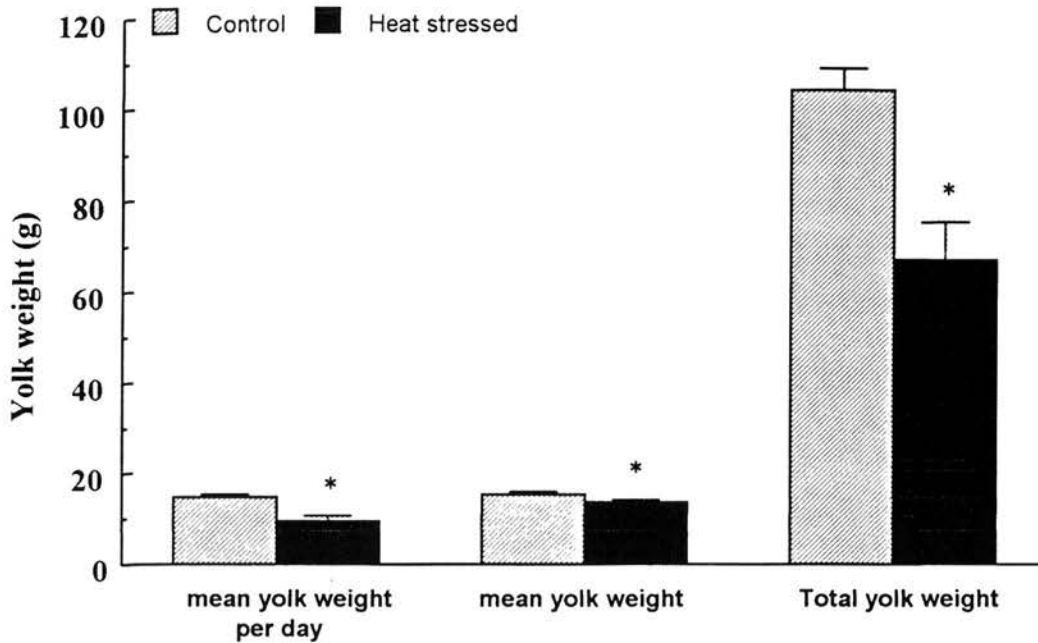


Figure 6.6. Effect of heat stress on mean yolk weight measurement (\pm s.e.m.) for control and heat stressed birds. * denotes significant difference ($P<0.05$).

6.3.2. Vitellogenin and VLDL Concentrations

Figure 6.7 shows that before chronic heat stress treatment, there was no difference in plasma zinc concentration between the two experimental groups. However, over the subsequent four weeks of treatment there was an overall significant reduction ($P<0.01$) in plasma zinc concentration in the chronically heat stressed birds, representing a 21% reduction in zinc concentration for the total four weeks of treatment. Similarly, examination of the differences in plasma triglyceride concentration between the control and heat stressed groups prior to, and at the end of the experiment showed that, like the plasma zinc concentration, there were no differences between the two groups.

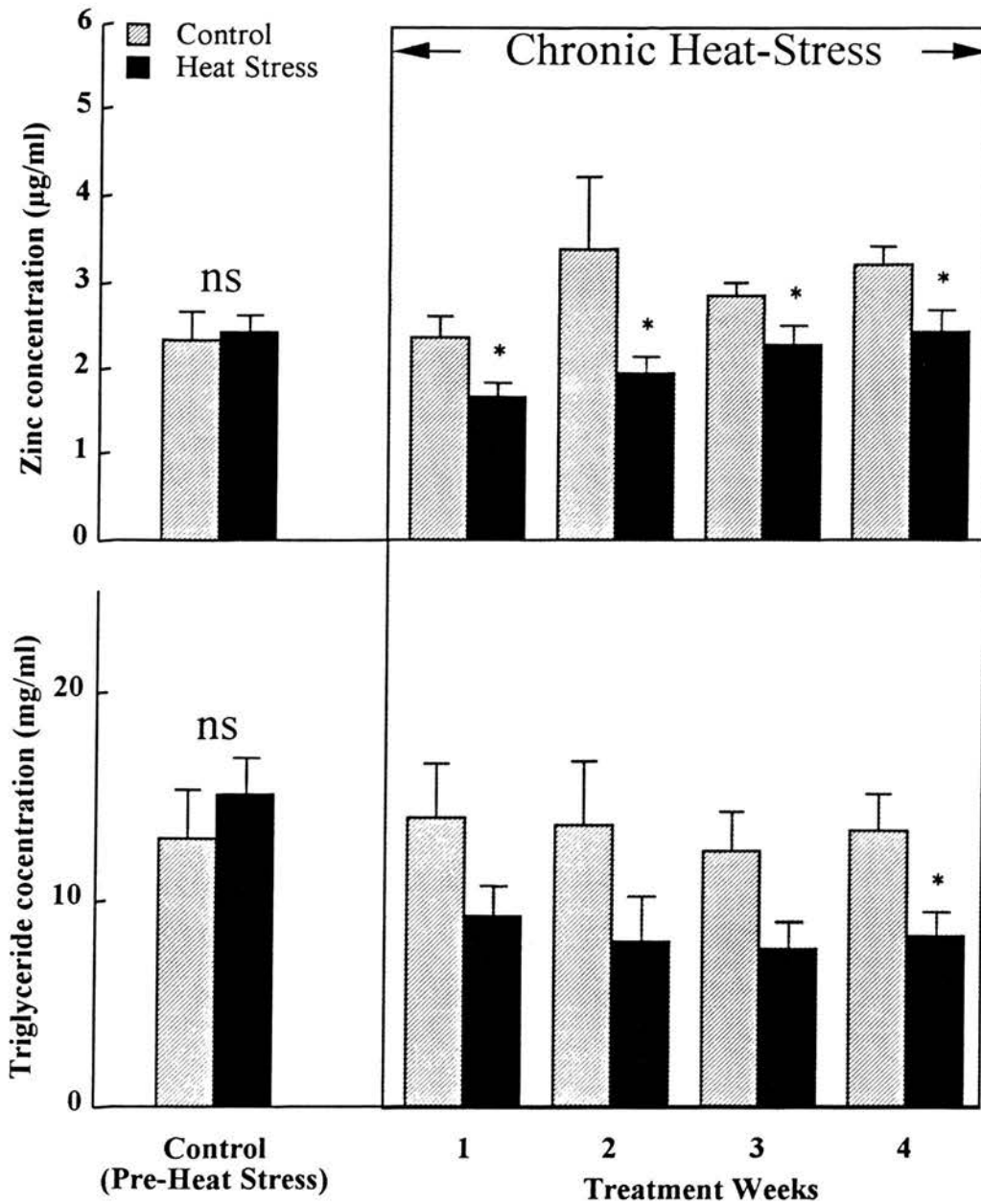


Figure 6.7. Plasma vitellogenin (Zn) and VLDL (triglyceride) concentrations (+ s.e.m.) for control and heat stressed birds. '*' denotes significant difference ($P < 0.05$).

During the first three weeks of the heat stress treatment there was a non significant reduction in plasma triglyceride concentration between the two groups, even though the data show a consistent numerical decrease of the same order as those observed for vitellogenin-zinc. Indeed, whilst the changes in plasma triglyceride did not reach significance, the heat stress induced depression was sustained longer than

that in plasma vitellogenin zinc. A significant difference was however observed after the fourth week of chronic heat stress exposure ($P<0.05$). However, over the four weeks of treatment there was a significant decrease (35%; $P<0.01$) in plasma triglyceride concentration in the chronically heat stressed group.

6.3.3. Chromatography by Gel Filtration

The elution profile of the standard was determined by working out the protein assay and matching with its molecular weights (**Table 6.2.**).

Table 6.2. Elution volume for each protein from the standard.

Proteins	Molecular weight (Da)	Elution volume (ml)
Thyroglobulin	0.67×10^6	77
Bovine γ -globulin	0.16×10^6	104
Chicken ovalbumin	44×10^3	111
Equine myoglobin	17×10^3	127

Blue dextran was eluted first and vitamin B₁₂ was the last, but they did not appear in the profile because they are not proteins. From the calibration of the column the approximate elution volume of vitellogenin could be predicted from its molecular weight.

Vitellogenin Purification

Approximately 4 ml of radiolabelled plasma were loaded into the column. All the fractions collected from the column were initially counted for radioactivity (**Figure 6.8**) and then were subsequently assayed for protein, zinc and triglyceride concentrations as shown in **Figures 6.9** and **6.10**.

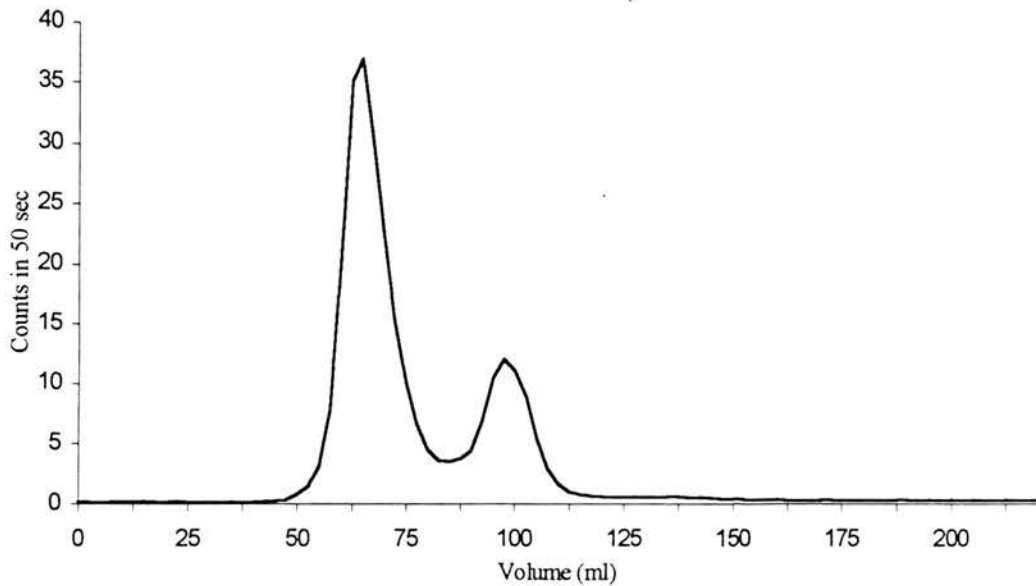


Figure 6.8. Radioactivity counts ($\times 10^3$) of initial fractions collected from the column.

In order to validate the flow rate and to calibrate the column this purification of vitellogenin was repeated several times in different experiments. The results of the study showed a similarity with the patterns of other experiments for both the initial counts and other plasma metabolite concentrations (see **Figure 6.10** for comparison).

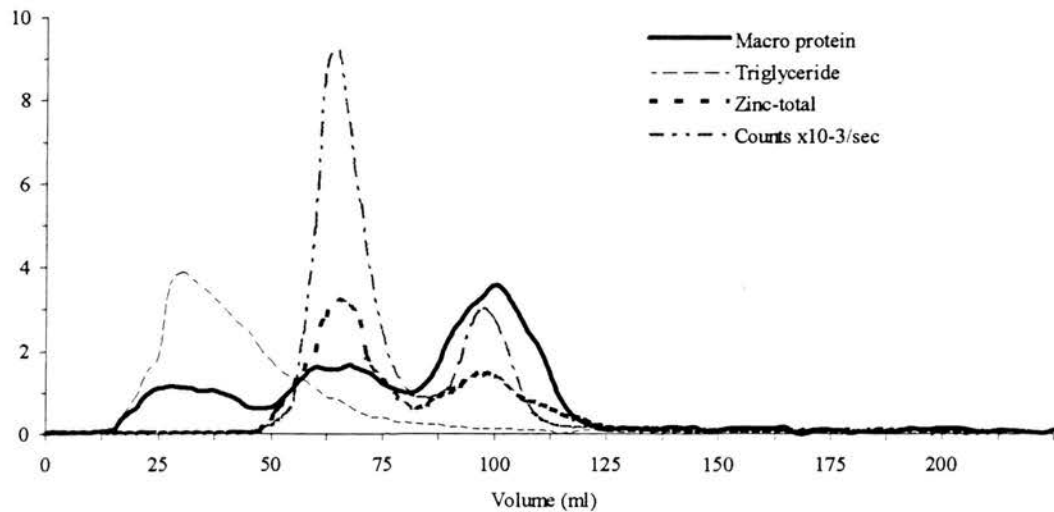


Figure 6.10. Another elution profile from other experiment within this study shows a similar pattern for the results of the metabolite plasma concentrations and their peaks. The peaks of macroprotein and the zinc were matched to the peak of the radioactivity counts.

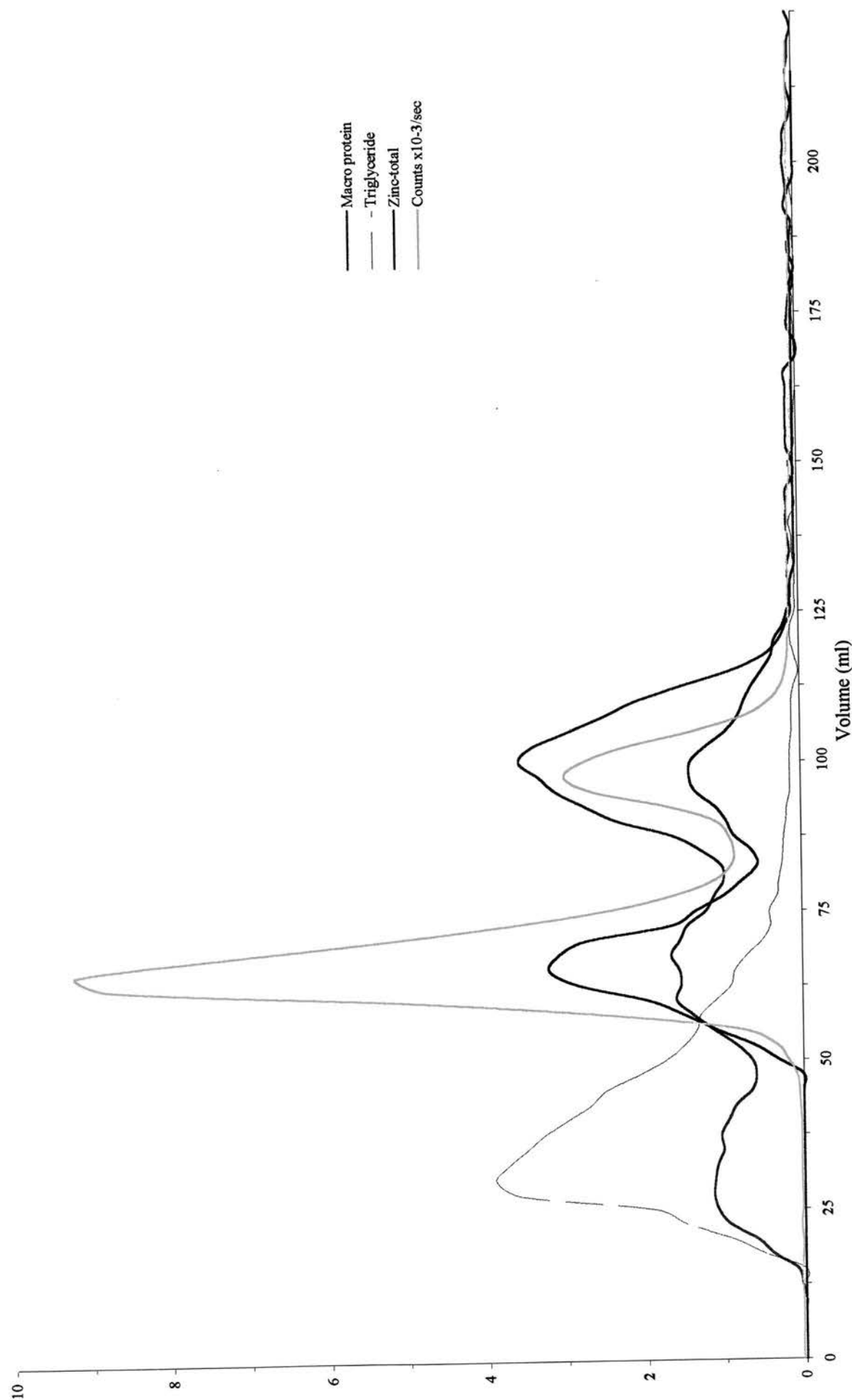


Figure 6.9. Elution profiles of plasma metabolites of protein, triglyceride, and total zinc plotted with the initial radioactivity counts.

Composite elution profiles in **Figure 6.9** show that the elution of the plasma yields three separate and identifiable protein peaks which are believed to represent fractions containing low density lipoprotein, vitellogenin and albumen. Confirmation of these hypotheses is shown in that nearly 90% of the plasma triglyceride elutes approximately over the same time course of that of the predicted low density lipoprotein fraction. Albumen has, as vitellogenin, a high affinity for zinc, and there is a competition between vitellogenin and albumen for ^{65}Zn binding. Vitellogenin was identified by two different ways (*a*) its protein peak (corresponds to zinc and radiolabelled zinc highest peaks) and (*b*) using the calibration profile and comparing the predicted molecular weight with that calculated from elution of the major zinc binding protein (**Figure 6.11**).

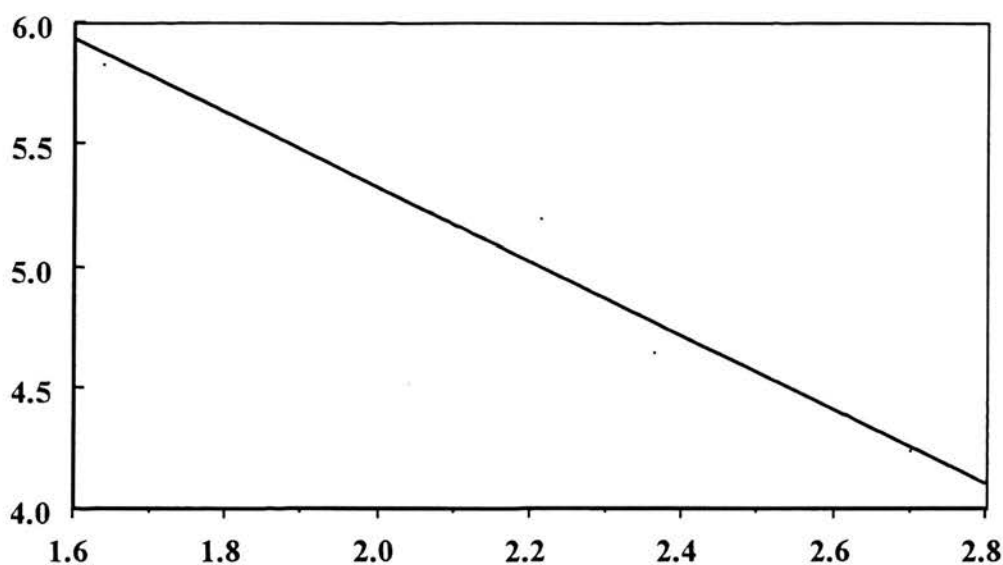


Figure 6.11. Determination of molecular weight, knowing V_e/V_0

Quality Factors and Values of Gel Chromatography Column

(a). Values obtained from the column calibration and from the purification:
 Height of the column (L) is 45 cm and width of the column (l) is 3 cm with a dead volume (V_0) of 50 ml. The total volume of the column (V_t) is 318 ml; so the volume of the gel in the column was yielded as $V_g = V_t - V_0 = 268$ ml. The elution volume of vitellogenin was (V_e) 89 ml.

(b). Volume range of plasma to load onto the column:

It has to turn out between 1 and 5% of the gel volume. The volume of plasma to load onto the column will have to be in a range of 2.7 ml to 13.4 ml.

(c). Partition coefficient (K_{av}):

Represents the proportion of pores that vitellogenin can fill in.

$$K_{av} = (V_e - V_o) / (V_t - V_o) = 0.14$$

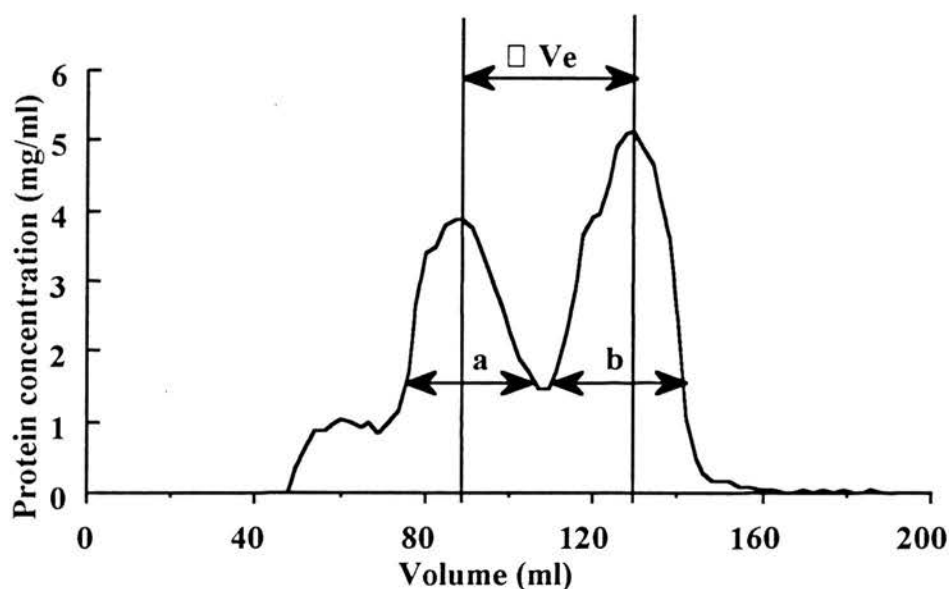


Figure 6.12. Determination of quality factors and values of the gel chromatography column.

(d). Resolution power (R_s):

Represents the power of the column to separate proteins.

If $R_s < 1$ there is no separation between the proteins,

If $R_s > 1.5$ the separation is effective.

$$a = 26.4 \text{ ml}; b = 30 \text{ ml}; \delta V_e = 40 \text{ ml}$$

$$R_s = \delta V_e / ((a + b)/2) = 1.4$$

(e). Number of theoretical plates (N) and Equivalent Height to a Theoretical Plate (EHTP):

They are defined for this column and for vitellogenin. EHTP is much more significant than N because it is relative to the height of the column.

$$N = (4 V_e/a)^2 = 181.8$$

$$\text{EHTP} = L / N = 0.2 \text{ cm}$$

The lower the EHTP value, the greater the column resolution power.

The dead volume of the column was obtained from the calibration: $V_o = 50$ ml. The elution volume of vitellogenin was obtained from the radiolabelled plasma elution profile: $V_e = 89$ ml. Using the linear regression equation the calculated molecular weight of vitellogenin was 457,210 Da, the molecular weight found in the literature for vitellogenin being 480×10^3 Da.

Centrifugal ultrafiltration

Bulked vitellogenin was analysed for protein and zinc, it was also counted for radioactivity (**Table 6.3**). After precipitation of vitellogenin with DexSO_4 the supernatant was analysed for protein. The results showed that protein was not found in the supernatant after precipitation of vitellogenin, therefore the protein concentration could be assumed is vitellogenin concentration. This allowed the calculation of the specific activity of vitellogenin using the calibrated coefficient for the counter (16.7 desintegrations correspond to a count per second). Specific activity of purified vitellogenin was 11.5 kBq/mg.

Table 6.3. Composition of the injected solution.

Radioactivity counts (sec/ml)	7292
Protein concentration (mg/ml)	10.55
Zinc concentration ($\mu\text{g/ml}$)	7

6.3.4. Administration and determination of radiolabelled vitellogenin uptake by the oocyte *in vivo*

Purified radiolabelled vitellogenin was injected (3.5 ml) into two birds (one from each experimental group). Blood samples were taken 2, 15, 30, 45, 60 and 75 min after injection and plasma was counted for radioactivity and protein, zinc and triglyceride assays. From the counts obtained from the blood and for each bird, curves were plotted (**Figures 6.13 and 6.14**) to evaluate the disappearance rate of ^{65}Zn from the blood and extrapolated in order to define the number of counts in the blood at the moment of injection. This allowed calculation of the total volume of blood in each bird (**Table 6.4**).

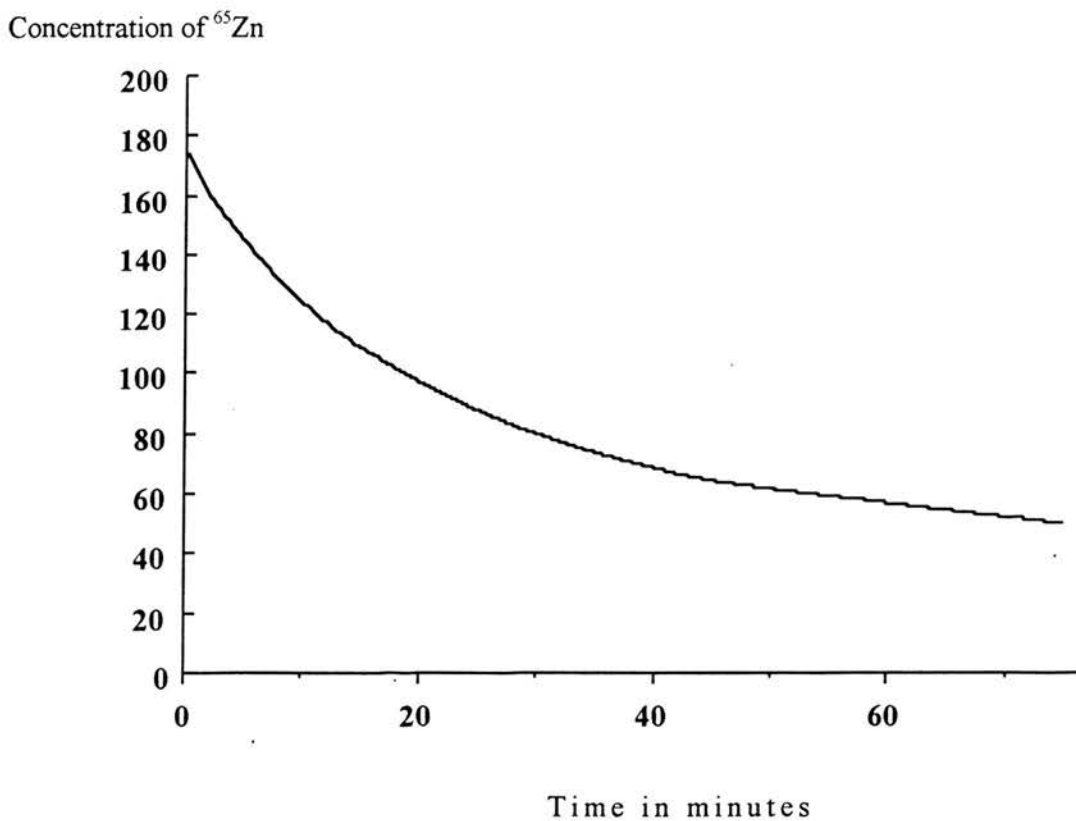


Figure 6.13. Elimination of zinc in the blood circulation of control bird

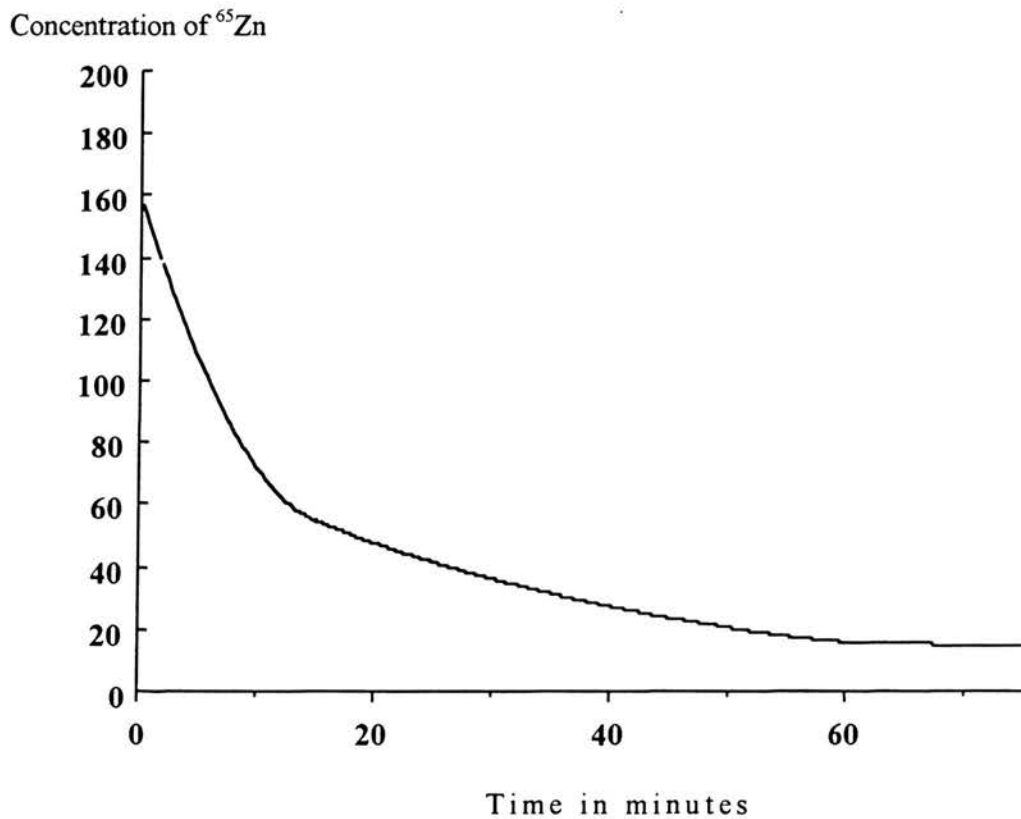


Figure 6.14. Elimination of zinc in the blood circulation of heat stressed bird..

Knowing the number of counts in the blood at the moment of injection for each bird and knowing the concentration of zinc in the plasma for both groups, the vitellogenin-zinc specific activity could therefore be determined (**Table 6.4**).

Table 6.4. Weight, blood volume and zinc concentrations of each bird .

Group	Weight (g)	Blood volume (ml)	Zinc concentration ($\mu\text{g/ml}$)	Vg-Zinc specific activity
Control	1980	146.7	4.5	38.7
Heat stress	1790	163.6	2.7	57.8

Blood samples were taken 2, 15, 30, 45, 60 and 75 mins post injection of labelled vitellogenin. Each blood sample was centrifuged to yield the plasma following the radioactivity counting. Plasmas obtained were then assayed for their protein, triglyceride and zinc concentrations and are shown in **Table 6.5**.

Table 6.5. Plasma metabolite concentrations and radioactivity after injection.

	Group	Time (min post-injection)					
		2	15	30	45	60	75
Counts/s/ml	C*	159.9	108.5	79.9	64.3	56.8	34.6
	HS	137.9	54.8	36.2	23.6	15.8	14.9
Protein (mg/ml)	C	66.0	63.8	68.2	68.2	66.3	67.0
	HS	69.6	75.0	76.1	67.0	69.1	69.1
Zinc (µg/ml)	C	4.5	4.5	4.1	3.9	3.9	3.5
	HS	2.7	2.4	2.3	2.0	2.3	2.2
Triglyceride (mg/ml)	C	23.9	23.4	21.8	22	20.1	21.6
	HS	6.7	6.6	7.5	6.9	7.0	6.8

* C: Control and HS: Heat stress

After 75 min, birds were killed and their oocytes and liver were removed, weighed and counted for radioactivity. From the blood samples obtained, plasma metabolites concentrations were assayed and zinc uptake by the liver and oocytes were calculated (**Table 6.6**) using vitellogenin-zinc specific activity of each bird (based on **Table 6.4**). Follicles were considered separately and as well as a unit of the hierarchy.

Table 6.6. Uptake rate of vitellogenin of each bird (mg/g/h).

	Oocyte	Liver
Control bird	1.71	2.36
Heat stressed bird	0.50	2.92
Effect of heat stress	-70.8%	+23.7%

The oocyte clearance could also be determined as representing the volume of plasma totally cleared by the oocytes which was 9.2 in control bird compared to 6.1 in heat stressed bird which means a 34% reduction. The vitellogenin uptake by the fast growing oocytes (follicles 1 to 5) in the hierarchy and in the liver were worked out by considering their counts and the content of zinc per organ, **Table 6.7** below.

Table 6.7. Vitellogenin uptake by the hierarchy of growing oocytes and the liver 75 min post injection.

	Weight (g)		Counts (cps/g)		μg of zinc per g		μg of zinc per organ		Equivalent mg of vitellogenin per organ	
	C*	HS	C	HS	C	HS	C	HS	C	HS
Follicles										
1	12.94	14.00	27.8	10.6	0.72	0.18	9.32	2.52	14.00	3.80
2	8.46	11.33	40.6	26.7	1.05	0.46	8.90	5.21	13.41	7.85
3	4.35	9.14	70.3	27.3	1.82	0.47	7.92	4.30	11.93	6.48
4	2.52	4.00	194.4	46.3	5.02	0.80	12.65	3.20	19.10	4.82
5	0.80	1.34	129.0	57.2	3.30	0.99	2.64	1.33	3.98	2.00
Hierarchy (Tot. fol.)	29.10	39.81	-	-	-	-	41.40	16.56	62.40	24.95
Liver	53.30	46.60	76.0	140.1	1.96	2.42	104.47	112.99	157.45	170.30

* C: Control; HS: Heat stress

The pattern of the clearance rate of the labelled vitellogenin in this study was similar and consistent with results obtained during the validation trials as shown in **Figures 6.15** and **6.16**.

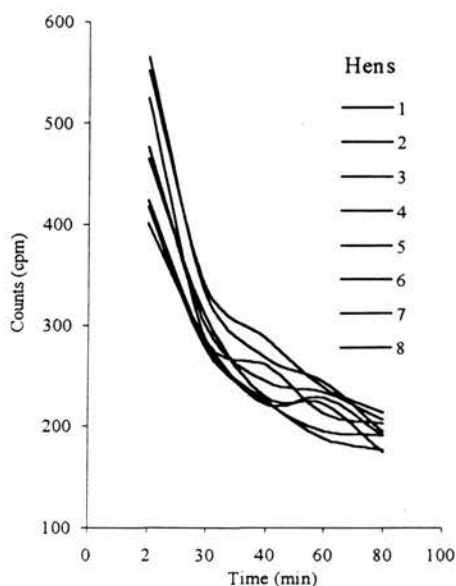


Figure 6.15.

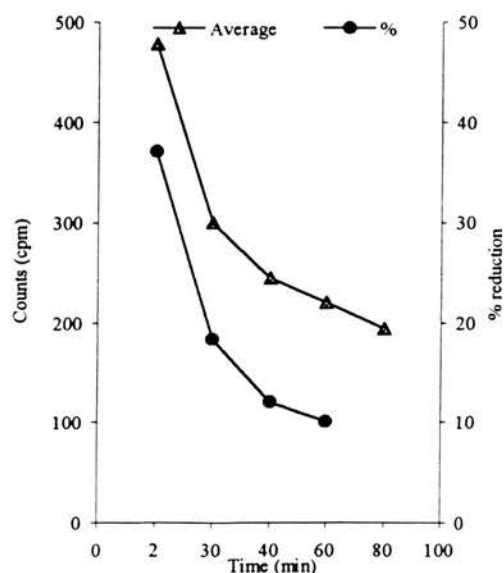


Figure 6.16.

Figures 6.15 and 6.16 show the clearance rate in the plasma of experiment 3 which are similar to results to the previous experiments on the pattern of circulating clearance rate ($n = 8$; Figure 6.15). The highest plasma clearance rate occurred during the first minutes post injection and became lower during the remain of blood samplings and had nearly disappeared by the end (80 mins; $n = 8$; Figure 6.16).

Knowing of the amount of radioactive labelled vitellogenin amount in the injected solution and quantifying the amount in the plasma, in oocytes and in the liver, the percentage of vitellogenin recovered at lost in other organs could therefore be calculated. The results are as shown in Table 6.8.

Table 6.8. Vitellogenin loss in other organs of each bird.

	Recovered	Unaccounted
Control bird	42.0%	58.0%
Heat stressed bird	38.9%	61.1%

6.4. DISCUSSION

In view of finding that the levels of LH and oestrogens, as well as other steroids, vary during the laying cycle and bearing in mind the hypothesis that the gonadotrophins may stimulate oocyte development by enhancing follicular transport mechanisms, it therefore seems possible that daily fluctuations in plasma vitellogenin may occur. Analyses of the data, however, indicate no consistent rhythm in the plasma concentrations of egg yolk precursors which could be related to position in the photoperiod, the clutch sequence or to ovulation.

The findings of this study do not support MacKenzie and Martin's (1967) and Redshaw and Follet's (1976) view that yolk uptake occurs at a constant rate throughout maturation, although they expected that there would be insufficient fine control over the system to prevent a sudden rapid increase in circulating vitellogenin just prior to and after ovulation of the most mature oocyte.

One of the possible explanations for the absence of a daily rhythm in plasma concentration is that the rate of protein uptake represents only a small fraction of the total amount circulating. Gruber (1972) has pointed out that some 3000 mg of protein is synthesised daily by the liver, about 50% of which is vitellogenin. That means at a constant rate of uptake this represents a loss from the plasma pool of only about 150 mg/h.

Heat stress results in a substantial decrease in egg production. The physiological responses to heat stress involve the functional integration of several organs to meet the metabolic needs of birds that are trying to dissipate heat which involves the regulation of the blood flow through the arterio-venous network acting as a heat exchanger (Midtgard, 1989). During exposure to a high ambient temperature birds try to dissipate heat as a first priority, this may lead to decrease in blood flow to the reproductive tracts as production of egg is less important than to maintain their survival.

Over the six weeks of treatment all the different indices of egg weight and egg production demonstrated a decrease in the heat stress group. All the findings can be concentrated in the comparison of the mean egg weight and the mean egg weight per

day, representing respectively the mean weight of egg produced and the theoretical mean egg weight if they had to be laid daily.

This comparison confirmed that much more time is required by a heat stressed bird to produce an egg. The reduction in mean egg weight per day in the heat stress group represents a 37% decrease compared to the control group.

In addition, a decrease in yolk production was also observed. Comparison between mean yolk weight and mean yolk weight per day showed the same response as for egg production. However, the difference between the two groups for the mean yolk weight per day represented a 35% reduction. It thus appears that exposure to heat stress reduces the number of eggs laid, the size or weight of each egg produced and the total yolk production and yolk per egg.

As the yolk size and total yolk production are decreased by heat stress these phenomena may be reflected in the synthesis and/or levels of the egg yolk precursors in the circulation. The results showed that both concentrations of vitellogenin and VLDL fell during heat stress. Although, the pattern of response for each precursor altered with the period of heat stress. **Figure 6.6** shows that as the VLDL concentration continuously fell, vitellogenin concentration fell initially during the first week then increased in the last three weeks towards the control values.

This observation could suggest that the two yolk precursors, even if synthesised under the same oestrogen action by the same organ, are synthesised by two different mechanisms with different response characteristics or control systems. It may be proposed that heat stress may inhibit yolk precursor synthesis in the liver either by reduction in oestradiol secretion or reduced sensitivity of the hepatocytes to oestrogen stimulation. Decreased precursor synthesis would account for the apparent reduction in plasma concentrations of vitellogenin and VLDL. The reduction in precursor availability may then result in decreased uptake by the oocyte, slower yolk accumulation and oocyte development and consequent changes in egg production. Adaptive responses in the oocyte uptake mechanisms may occur concurrently during heat stress but have yet to be characterised.

The primary aim of the present study was the development and validation of a technique to quantify egg yolk precursor uptake by oocytes *in vivo* in the laying hen.

The labelling of natural vitellogenin present in high concentrations in fresh laying hen plasma by incubation with ^{65}Zn proved extremely effective. Chromatography on an A5-agarose gel indicated that 60-70% of radioactive zinc binding occurred in a protein peak corresponding to the predicted elution volume and molecular weight of vitellogenin (**Figure 6.7**).

The majority of the remaining ^{65}Zn binding eluted with a protein likely to be serum albumin. The analysis of column eluents for triglyceride, zinc and protein confirmed separation of vitellogenin from VLDL and other potential zinc binding proteins and indicated protein rich fractions of high zinc content and ^{65}Zn specific activities.

Bulking of such fractions and subsequent concentration of the protein components by selective centrifugal ultrafiltration yielded a ^{65}Zn -vitellogenin preparation. This was characterised by a protein concentration of 10.55 mg/ml and a zinc concentration of 7 $\mu\text{g/ml}$ and a measured radioactivity of $440 \times 10^3 \text{cpm/ml}$. It was thus possible following correction for counting efficiency (6%) to determine the specific activity of the vitellogenin preparation. Treatment of this material with dextran sulphate-magnesium chloride (Griffin and Mitchell, 1983) confirmed that all zinc radioactivity, zinc and protein were precipitable, thus confirming the high purity of the preparation.

It was thus possible to inject this vitellogenin preparation at known doses into a control and a heat stressed bird and monitor its distribution, elimination from the plasma, uptake by the liver and oocytes and effective recovery by means of γ -counting of the appropriate samples.

From the elimination rates it was possible to determine effective blood volume and 'time 0' radiolabelling level in the plasma by extrapolation. In turn, this could be related to the corresponding plasma vitellogenin-zinc concentration. Further calculations allowed calibration of the quantity of radioactivity taken up by an organ in terms of vitellogenin-zinc and finally vitellogenin protein. The apparent recovery of ^{65}Zn -vitellogenin in these preparations of approximately 50% is more than adequate for this type of preparation. Oocyte vitellogenin accumulation rates in the control and heat stress bird could thus be compared on a valid basis.

The mechanism of Zn elimination is, however, not fully understood, although it has been demonstrated that zinc metabolism is controlled at least by two homeostatic mechanisms that act at the sites of absorption and of intestinal excretion (Cotzias and Papavasiliou (1964).

The results obtained from the oocytes and liver measurements following labelled vitellogenin injection have to be carefully viewed with caution as the experiment only involved two birds and that the calculations rely upon some basic assumptions. Even though the differences found between two treatments in this experiment, it is suggested that future investigations are undertaken on a much larger sample size.

As found in the plasma results, the amount of vitellogenin uptake by the oocyte is greatly reduced in the heat stressed bird. This represent a 70% reduction compared to the control bird. The difference in the plasma concentration for vitellogenin represented only a 21% reduction in the heat stressed bird. The greater reduction in uptake than in plasma concentration may be explained by a change in blood flow to the oocyte or in yolk membrane uptake capacity or function. Changes in blood flow to the reproductive tract appear as soon as the bird suffers from heat stress. In order to increase exchanges of heat with the external environment blood flows to cutaneous capillaries. This phenomenon requires a large volume of blood, only vital organs are perfused and the reproductive apparatus represents a lower priority. This may explain the 34% reduction of clearance of vitellogenin observed in the heat stressed birds.

The recovery quantifies the amount of vitellogenin ⁶⁵zinc that was found in organs and plasma expressed as a fraction of the amount of injected ⁶⁵Zn-vitellogenin. A large part of the lost ⁶⁵Zn-vitellogenin may have been transported to other tissue compartments or organs by the blood circulation.

Various other factors may also affect Zn elimination and should perhaps be considered as potential areas (McLean and Speakman, 1995) for control to improve the technique. Over than 30 years ago Zeigler et al. (1964), found that elimination of ⁶⁵Zn was lower in zinc-deficient animals than in zinc-sufficient animals. Whilst diets of low zinc and high calcium in dogs produced higher elimination rates of Zn in faeces

compared with control as reported by Robertson and Burns (1963). The effects of food intake on elimination rate in both these studies were not reported.

The findings indicate that the technique is certainly appropriate for the measurement of oocyte vitellogenin uptake *in vivo* and therefore for the elucidation of the mechanisms controlling oocyte development and yolk accumulation in response to environmental challenges. The study has clearly achieved its primary objectives and will form the basis for more thorough validation and improvement of the techniques, which will then constitute an important avenue for progress in the on-going wider programme relating to egg production in tropical climates.

These considerations suggest that the technique may not be of particular use in the field, and further validation work would be required to establish its usefulness in any specific circumstance.

This also considered necessary as oestradiol may control not only hepatic yolk precursor synthesis but also the uptake process at the oocyte. It is suspected that during heat stress there may be oestrogen deficiency in birds. This has been indicated by the evaluation of the effects of oestradiol administration which induced the availability of egg yolk precursor in the circulation. Yet the role of oestrogen on the transport and uptake of vitellogenin and VLDL during heat stress may merit further investigation.

CHAPTER SEVEN

AN INVESTIGATION OF THE ROLE OF DIETARY VITAMIN E IN ALLEVIATING THE EFFECTS OF HEAT STRESS IN LAYING HENS

7.0. INTRODUCTION

Incorporation of vitamin E in the diet may help to reduce lipid peroxidation. The active form of vitamin E, α -tocopherol, interacts with selenium-containing glutathione-peroxidase to prevent the oxidative damage. Since heat stress may cause in tissue damage, dietary supplementation of vitamin E can be expected to help in maintaining the cell membrane of tissues. It, therefore the present study carried out in this **Chapter Seven** was to confirm the effects of heat stress on the plasma egg yolk precursor levels (vitellogenin and VLDL) and especially to determine the influence of vitamin E supplementation upon this process during chronic heat stress.

7.1. MATERIAL AND METHODS

The experiment was carried out for five weeks and was divided into three experimental periods, pre-heat stress (adaptation) (22°C, two weeks), heat stress (heat stress) (35°C, one week) and post-heat stress (recovery) (22°C, two weeks) periods all with a relative humidity (RH) 35%.

Experimental birds

Twenty-four 35 week-old ISA brown laying hens were used in this experiment divided randomly into two groups, Control and Vitamin E supplemented. They were

caged individually in modules and housed in a temperature-controlled chamber. Chamber temperatures and relative humidities (RH) were recorded every five minutes throughout the experiment by a Squirrel Meter/Logger model 1200. Prior to the laying period the birds were kept individually caged under a 24 h artificial lighting pattern (14L:10D photoperiod; lights on at 06:00-h GMT). Egg laying records were kept for each hen and only hens which had a good laying record were selected for further study. The hens used in any one study were of the same batch. On the day of the experiment, birds were transferred to the controlled-climate chambers, caged individually and exposed to the specified temperatures and relative humidities. At the end of the study birds were killed by a lethal dose of phentobarbiturate (Rhône-Mérieux, Southampton, UK) injected intravenously through the brachial vein.

Controlled climate chamber and cages

A controlled climate chamber (3.5 x 2.0 x 2.0 m) was used. The chamber could be maintained at different climatic conditions. Temperature could be controlled within the range -5 to +40°C ($\pm 0.2^\circ\text{C}$) and the relative humidity (RH) between 10 and 100% ($\pm 5\%$). The temperatures and relative humidities of the chamber were measured continuously throughout the experiment and recorded every 5 min by a Data Logger (1200 Series Squirrel Meter/Logger, Grant Ltd., UK). The data recorded by the Logger were transferred to a PC by 20/20⁰ programme. Each cage was equipped with an individual feeder, which allowed measurement of individual food intake, and an automatic nipped-drinker. Each controlled-climate chamber had the same design and lighting regime.

The food and vitamin E supplementation in the diet

The control diet was a standard layer's mash containing 160 g crude protein, 11 MJ metabolisable energy and 33 g calcium/kg. The experiment diet was mixed of the control diet supplemented with 500 mg of vitamin E per kilogram diet. The vitamin E used in this trial was DL α -tocopheryl acetate as powder with an activity of 500 IU/g, absorbed on expanded silica). The food and water were provided *ad libitum*.

Body temperature and food intake monitoring

The measurements of deep body temperature were performed within the climate chamber to avoid any changes in body temperature. An electronic rectal probe (thermometer model RS-612-849) was used to measure the deep body temperature by inserted approximately 5 cm into the bird's rectum. The mean values of body weight, rectal temperature, food intake and food conversion efficiency were calculated using Minitab Release 10 (Minitab Inc., UK). The birds' body temperatures were measured twice a week at the same time as of blood samplings were taken to minimise the stress of handling..

Collection of blood samples

Blood samples (about 2.5-3.0 ml) were withdrawn into heparinised (heparin in physiological saline 50 units/ml) syringes from the brachial vein and immediately transferred to heparinised tubes (3 ml tube - Teklab). The blood was placed on a roller (Spiramix 5 roller, Jencons (Scientific) Ltd.), and then centrifuged at 1500 g for 10-min (Denley BS 200 centrifuge) to obtain the plasma fraction. Plasma was stored at -20°C for pending analysis.

Plasma metabolites determination

Vitellogenin was measured as plasma zinc (Zn) and very low density lipoprotein (VLDL) as plasma triglyceride as described by Mitchell and Carlisle (1991). Calcium (Ca) was measured by the in vitro-OCPC method for the quantitative determination of total plasma calcium. Plasma triglycerides (TG) were measured by the method of glycerol-3-phosphate-oxidase peroxidase. Data were analysed by ANOVA-General Linear Model. All assays, i.e. total zinc, VLDL depleted zinc, VLDL and alkaline phosphatase activity were modified and previously validated for use in avian systems and performed by an automated plate reading spectrophotometer as described in the **Chapter Two: General Materials and Methods**.

Egg production

Eggs were collected individually and weighed on a daily basis from 10.00 to 18.00h. The eggs collected on that day were assumed to be the egg production for that group on that specified day. Any broken eggs collected were regarded as missing data for the egg and yolk weights but they were included in the egg production records. After taking the egg weights the eggs were broken and the albumen was separated to obtain the yolk weights. In order to have a constant weight egg shells were dried overnight in the oven at 60°C. On the following day the dried shell, together with the very thin layer of adhered membrane was weighed. The shell thickness was measured using a micrometer and recorded three times at different sites on the shell, i.e. at the middle and at two poles of the shell and a mean thickness calculated.

7.2. RESULTS

As summarised in **Table 7.1** heat stress reduces egg production in terms of egg number and total egg mass produced. Vitamin E treatment significantly increased both vitellogenin (+44%) and VLDL, as plasma triglyceride (+44%) concomitant with increases in egg production (+41.9%), yolk mass per egg (+7.1%) and total yolk mass (+28.6%).

Table 7.1. Effect of dietary vitamin E supplementation and heat stress on egg production changes in laying hens.

	Adaptation	Heat Stress	Recovery
\bar{x} egg weight (g)	C 66.0 ^a E 68.7 ^a (+4.1%)#	C 63.1 ^a E 66.6 ^a (+5.5%)	C 63.6 ^a E 67.6 ^a (+6.3%)
Σ egg numbers (n)	C 52 ^a E 57 ^a (+9.6%)	C 43 ^a E 61 ^b (+41.9%)	C 40 ^a E 60 ^b (+50%)
Σ egg mass (g)	C 3435 ^a E 3915 ^b (14%)	C 2717 ^a E 4064 ^b (+49.6%)	C 2538 ^a E 4054 ^b (+59.7%)
Σ egg weight/bird (g)	C 40.9 ^a E 46.6 ^b (+13.9%)	C 32.3 ^a E 48.3 ^b (+49.5%)	C 30.2 ^a E 48.3 ^b (+59.9%)
\bar{x} yolk mass per egg (g)	C 18.70 ^a E 19.50 ^a (+4.2%)	C 17.66 ^a E 18.92 ^b (+7.1%)	C 17.81 ^a E 18.45 ^a (+3.6%)
Σ yolk mass (g)	C 972.4 ^a E 1111.5 ^b (14.3%)	C 759.4 ^a E 1154.1 ^b (+52%)	C 712.4 ^a E 1107 ^b (+55.4%)
\bar{x} shell mass/egg (g)	C 6.64 ^a E 6.83 ^a (+2.9%)	C 6.07 ^a E 6.53 ^b (+7.6%)	C 6.33 ^a E 6.67 ^a (+5.4%)
Σ shell mass (g)	C 464.9 ^a E 573.7 ^b (23.4%)	C 425.1 ^a E 548.5 ^b (+29.0%)	C 487.4 ^a E 560.3 ^b (+14.9%)

Σ : total; \bar{x} : mean # : response to high concentration vitamin E supplementation.
Means in each column followed by different superscripts differ significantly ($p < 0.05$).

In this experiment the circulating level of vitellogenin, as plasma zinc (Zn) concentration, was only slightly changed in the vitamin E group between the adaptation and the heat stress periods, but a significant reduction was observed in the control (untreated vitamin) group ($p<0.01$; **Figure 7.1**). Therefore, a highly significant difference was observed between groups during the heat stress period ($p<0.001$); it seemed vitamin E supplementation was capable of preventing a big reduction of vitellogenin in the plasma level.

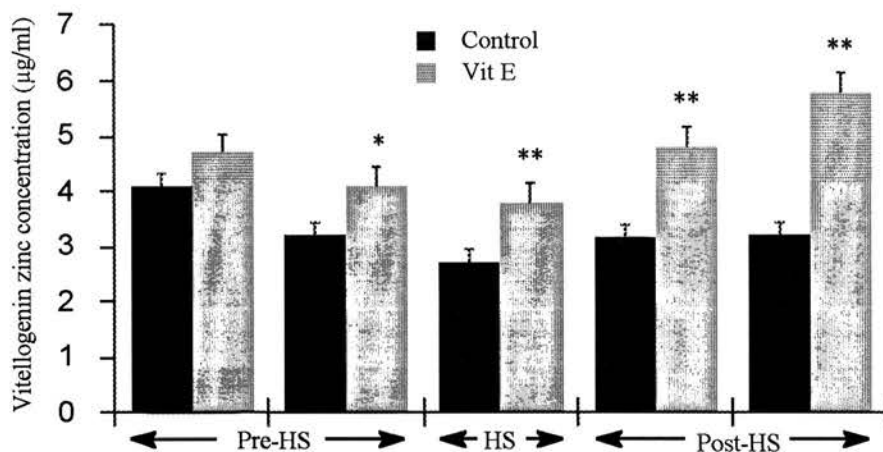


Figure 7.1. Effects of vitamin E supplementation and heat stress on plasma zinc (vitellogenin) concentration (weekly means + SEM)

* in comparison between two groups denote a significant difference ($p<0.05$)

** as highly significant different ($p<0.01$).

Although the pattern of changes in the circulating level of VLDL was different with that of vitellogenin, vitamin E also increased the availability of its concentration during heat stress in comparison to the untreated (control) group. There was no difference observed between adaptation and recovery periods in both groups ($p>0.05$), but a significant increase in VLDL concentrations was observed during the recovery period in both groups ($p<0.05$; **Figure 7.2**). Plasma concentrations of VLDL were slightly reduced in the vitamin E group in the same period, but the reduction was less severe and returned to normal after the heat stress period earlier than those in the control group.

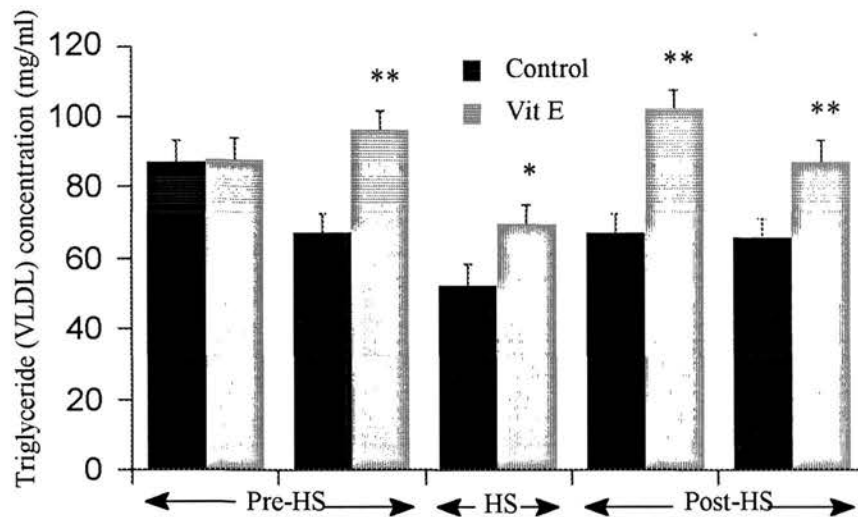


Figure 7.2. Effects of vitamin E supplementation and heat stress on plasma Triglyceride (VLDL) concentration (weekly means + SEM)

* in comparison between two groups denote a significantly different ($p < 0.05$)

** as highly significant different ($p < 0.01$)

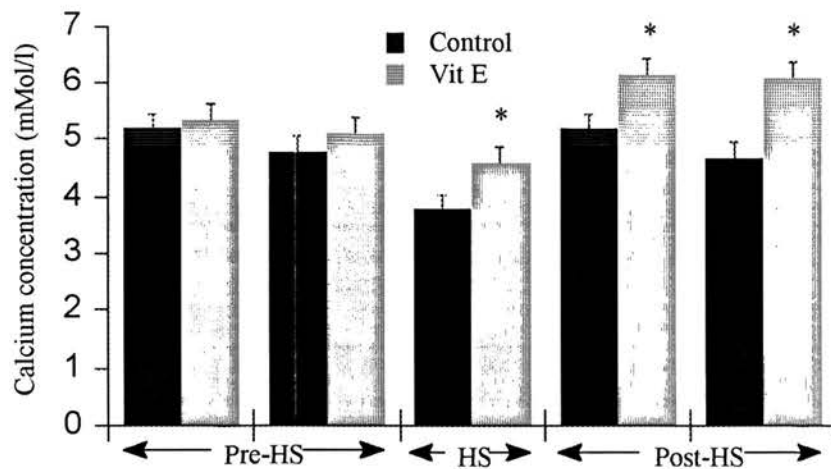


Figure 7.3. Effects of vitamin E supplementation and heat stress on plasma calcium concentration (weekly means + SEM)

* in comparison between two groups denote a significantly different ($p < 0.05$).

Table 7.2. The effect of vitamin E supplementation and heat stress in changes in food intake within groups (g).

Group	Adaptation		Heat Stress		Recovery	
	Mean	s.e.m.	Mean	s.e.m.	Mean	s.e.m.
Vitamin E	154.8 ^a (100%)	3.77	118.4 ^b (76%)#	9.38	140.6 ^a (91%)	9.23
Control	164.6 ^a (100%)	9.80	106.6 ^b (65%)	3.48	144.9 ^a (88%)	6.62

Means in the same rows with different superscripts differ significantly ($p < 0.05$)

#: changes in food intake compared to the adaptation within group.

Table 7.3. The effect of vitamin E supplementation and heat stress on changes in body temperature within groups (g).

Group	Adaptation		Heat Stress		Recovery	
	Mean	s.e.m.	Mean	s.e.m.	Mean	s.e.m.
Vitamin E	41.12 ^a	0.04	42.04 ^b	0.03	41.70 ^a	0.05
Control	41.02 ^a	0.02	41.98 ^b	0.09	41.70 ^a	0.03

Means in the same rows with different superscripts differ significantly ($p < 0.05$)

7.3. DISCUSSION

Vitellogenin and VLDL are the major yolk precursors in the plasma of the laying hen and are synthesised in the liver in response to oestrogen stimulation. It is also well known that the quantity of yolk produced is a major factor in the determination of the ultimate size of the egg (Griffin *et al.*, 1984, Redshaw and Follett, 1972). Heat stress may lead to cellular damage due to over production of oxygen free radicals, which cause lipid peroxidation in the cell membrane, especially in high metabolic activity organs (Slater, 1984; Fowler, 1990). If cellular damage is reduced by the antioxidant effect of vitamin E in tissues, e.g. in the liver and ovary, then egg yolk precursor synthesis and incorporation in to the ovarian follicle may be less affected by heat stress conditions. The unchanged level of egg production observed throughout the experiment in the vitamin E group, in particular during the heat stress period, is probably due to a maintained production of egg yolk precursors in the liver.

Plasma concentrations of calcium were significantly reduced during the heat stress period in vitamin E group ($p < 0.05$) and to a greater degree in the control group ($p < 0.01$; **Figure 3**). A significant increase in plasma total calcium (Ca) concentration was observed during the recovery period in both groups ($p < 0.05$). No difference was observed during the heat stress period between groups but a significant difference was recorded during the recovery period ($p < 0.05$).

The dietary vitamin E supplementation has been able to help heat stressed hens to maintain their egg production has been confirmed in subsequent trials (Utomo *et al.*, 1993; 1994). Similar responses have been also reported by De-Andrade *et al.* (1977), and related to respiratory alkalosis during heat stress reducing blood ionised Ca which may in turn limit the availability of Ca for eggshell formation (Odom, *et al.*, 1986). The present results may indicate a greater availability of calcium for shell production during heat stress in birds receiving vitamin E supplementation. This may be reflected in the higher total shell mass produced in this group (+29%; **Table 7.3**). Miller and Sunde (1975) have suggested that Ca metabolism in heat stressed laying hens may be impaired by a reduction in thyroid hormone and calcitonin production and secretion in such a way that they are maintained in balance but at lower concentrations. In the present study there were no

significant differences in total egg mass produced per day during the adaptation period between groups, but a significant reduction ($p < 0.02$) during the heat stress period in the control group. In the vitamin E group egg production increased during the recovery period ($p < 0.001$; **Table 7.3**).

Inclusion of vitamin E in the diet resulted in an important increase in egg production throughout the experiment. Vitamin E supplementation resulted in an increment of average egg weight (+5.5%), total egg number (+41.9%), daily rate of egg mass production (+46.6%), and total egg weight production per bird (+49.5%), in comparison with the control group (**Table 7.3**). The present findings are at variance with those of Richter *et al.* (1986), who reported that low level supplementation of selenium (Se) and vitamin E (20mg/kg) did not influence food intake, laying performance and food efficiency. The difference in the dosage of vitamin E supplemented to the diet, which was 25 times in this study compared with those of Richter's work, might be the answer. It possibly requires a high level of supplementation of vitamin E to initiate a such improvement.

Heat stress significantly reduced the feed intake in both groups, but more severely in the control group ($p < 0.001$; **Table 7.1**) and significantly increased the body temperature to the same extent in both control and vitamin E supplemented birds ($P < 0.05$; **Table 7.3**). Vitamin E treatment increased ($p < 0.05$) the food intake during the heat stress period. Suppression of food intake to avoid hyperthermia during exposure to heat stress is a recognised thermoregulatory strategy in domestic fowls. Similar responses have been observed by many workers including Emery *et al.* (1984) and de-Andrade *et al.* (1976), who reported a reduction in food consumption of 25% when birds were exposed to high ambient temperatures (32°C). Reduced food intake which in turn reduces net energy is probably a major contributing factor in decreased egg production at high temperature.

Vitamin E promotes the production of thyrothropic and adrenocorticothropic hormones and the gonadotrophins, whereas in vitamin E deficiency the hormone content of the pituitary falls (Rôche, 1976). If it is, vitamin E may improve hormonal functions, particularly during stressful conditions, and thus egg yolk precursors synthesis. High levels of vitamin E supplementation during heat stress resulted in a significant increase in total egg mass production, egg weight, total number of eggs produced and total egg weight per

bird in comparison to the control heat stress group. Since it has been demonstrated that α -tocopherol is a super chain terminator of free-radical chain reactions (McCay, 1985), it may be proposed that the positive response observed in the vitamin E treated group was in part due to reduction in cell damage which often follows heat stress. Vitamin E, with its antioxidant properties that maintain the membrane integrity, may mediate improvements in endocrine function, hepatic synthesis of egg yolk precursors and calcium homeostasis in heat stressed fowls.

The wide range of metabolic responses result in alterations in the circulatory profiles of a number of metabolites and intracellular constituents including enzymes. It has frequently been observed that physiological changes in various organ systems alter the limiting membrane integrity and result in the release in the blood plasma of enzymes normally located within the intracellular compartment into outside the cell (White, 1963; Combs *et al.*, 1975). Vitamin E, through its intra-membrane antioxidant properties, may protect the tissue membranes from lipid peroxidation due to free radical attack and minimise the associated loss of integrity of function and associated increased permeability.

These results suggest that vitamin E supplementation may improve the availability of egg yolk precursors and hence egg production during exposure to high thermal loads and mechanisms of this action may involve a reduction in possible heat stress induced oxidative damage to cellular components and tissue.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

The works and the findings presented in this thesis were designed to determine the physiological responses and to evaluate some of the mechanisms involved during the exposure of laying hens to chronic high ambient temperatures, which causes heat stress conditions in the domestic fowls. The findings in the first part of this thesis (**Chapter Three**) show that chronic exposure of laying hens to high ambient temperature causes several complex physiological changes. These changes include increased deep body temperature, reduced yolk and egg production concomitant with reduced plasma concentrations of the two major egg yolk precursors, vitellogenin and triglyceride-rich very low density lipoprotein (VLDL), and decreased food intake. In addition, the circulating levels of plasma metabolites such as total- and ionised calcium and inorganic phosphorus decreased whilst blood pH and plasma alkaline phosphatase increased.

Exposure the hens to high ambient temperatures causes a condition that is commonly defined as a heat stress condition. The heat-stressed birds try to cool down their body temperature by dissipating the excessive heat. When the capability to dissipate the heat is less than the heat accumulated in the body, the birds start panting and their body temperature begins to increase. Sensible heat loss can be enhanced by increasing blood flow to the skin, particularly the legs and feet, and by keeping these structures and the whole body, if that is possible, in a shade and in a flow of air (Hillman *et al.*, 1985; Phillips *et al.*, 1985). While the heat-stressed bird is panting, blood flow to the upper respiratory tract is increased, blood flow to the evaporative regions is more than doubled, whilst blood to some other parts of the body is reduced (Akester, 1984). In the terminal stages of hyperthermia cardiac output also decreases. Thus a contributing cause of death in the bird in extremely high temperatures is circulatory failure (Hafez, 1968).

It is established that the two major egg yolk precursors (vitellogenin and triglyceride rich-VLDL), which are synthesised by the liver, are the main materials required for egg yolk formation. The availability of these precursors in the plasma is vital for growth and development of the oocytes. Any conditions causing a reduction in their availability may lead to disturbances in egg production. Decreased precursor synthesis would account for the apparent reduction in plasma concentrations of vitellogenin and VLDL.

Chronic heat stress in this study has shown affecting egg yolk precursors' concentration in the blood circulation, with or without the decrease of its synthesis rate. These changes may be the main reason for decreased egg yolk produced by heat-stressed hens. There was a strong positive correlation between changes in vitellogenin and VLDL concentrations in the circulation in the domestic fowls reared under thermoneutral condition (Mitchell and Carlisle, 1991). This correlation, however, is altered in heat-stressed hens. This phenomenon leads to an assumption that secretion of these two precursors may be linked together but are controlled by different mechanisms, in conjunction to responses to heat stress, under the influence of the oestrogen. In addition, analysis of the data may also indicate no consistent rhythm in the plasma concentration of egg yolk precursors which could be related to position in the photoperiod, the clutch sequence and/or to ovulation (Guyer *et al.*, 1980; Wahli *et al.*, 1981). Therefore, a daily fluctuation in the plasma vitellogenin and VLDL may occur (Deeley *et al.*, 1975).

Although the factor(s) controls the exact mechanism in the synthesis of egg yolk precursors in heat-stressed hens remains poorly understood, it can be suggested that different findings reported in egg production and plasma egg yolk precursor concentrations in heat-stressed hens may be due some factors involved. They are: a) different dry bulb temperatures to induce heat stress; b) different duration of heat stress; c) different heat loads (i.e. the combination of dry bulb temperature and humidity) to induce heat stress; d) different ages of birds; e) different strains of birds; f) different diets fed to birds; and/or g) different degrees of thermotolerance.

8.1. Major contributions of the thesis

Only a few studies attempt to explain the actual physiological mechanisms involved in laying hens, especially those relating to the changes in vitellogenin and triglyceride rich-VLDL secretion and their deposition in the growing oocytes. In addition, the effects of heat stress on egg yolk precursors and their uptake by the growing oocytes are still poorly understood. In this study the effects of different thermal stresses on laying hens have been investigated by a series of experiments to measure the effects of these stresses on physiological and laying changes.

The works in **Chapter Three** confirmed that heat stress reduces yolk and egg production and daily yolk deposition concomitant with reductions in the circulating two major precursors, vitellogenin and VLDL. The decreased plasma concentrations of egg yolk precursor in heat-stressed hens are proven to be not mainly due to reduced food intake (**Chapter Five**). During the period of food restriction the reduction in egg production was less than proportional to the reduction in food intake, however the reduction in egg yolk precursors in the blood was more than proportional to the reduced food intake. This is confirmed by the works of feeding restrictions (85, 70 and 55% of normal food intake) compared with control group (*ad libitum*; 100%) and of a pair-feeding which showed that reduced food intake in heat-stressed hens contributed 'only' to some extent to the reduction in egg production. Hens with up to 30% food restriction (Groups 1 and 2) had a better weekly egg output (98% and 84% egg production in week 1 and 2 for Group 1; and 93% and 67% egg production for Group 2) compared to those in heat-stressed hens in the previous studies (91% and 77% egg production in week 1 and 2) with similar food reduction; whilst the pair-fed hens showed a better egg output (103% and 88% egg production in week 1 and 2) than heat stressed hens.

In addition the food restriction treatment to 55% did not significantly affect egg weight and yolk size throughout the experimental periods as shown in **Figure 5.7** above. In the previous studies (**Chapters Three and Four**) heat-stressed hens which voluntarily

voluntarily reduced food intake showed decreases in egg weight and yolk size (around 8% reduction) and approximately a 35% reduction in 'total' weekly egg mass.

These studies demonstrated that the reduction in food intake was less than the decrease in yolk and egg production, and plasma concentrations of yolk precursors.

Some authors suggest that heat stress decreases yolk and egg production probably by reduction in oestradiol secretion and/or reduced sensitivity of the hepatocytes to oestrogen stimulation. These matters were investigated in the studies reported in **Chapter Four**. The effects of oestrogen administration on egg yolk precursor changes and egg production in heat-stressed hens were evaluated. The results showed that the liver of the laying hen is still capable of synthesising vitellogenin and VLDL during heat stress. This was demonstrated by oestrogen challenges that increased the availability of egg yolk precursors in the circulation of heat-stressed hens. However, hepatocyte sensitivity to oestrogen stimulation is probably altered during heat stress; and/or the availability of oestrogen in the circulation is reduced during heat stress either due to a blockage in its synthesis or shortage in its life span (the rate of oestrogen catabolism is higher than its anabolism). In addition, interesting phenomena were found in the VLDL results in this study. Oestrogen administration under a thermoneutral environment increased VLDL concentration in control hens but only caused a small response in heat-stressed hens. This finding has led to an assumption that the two yolk precursors, although synthesised under the same oestrogenic action by the same organ, are synthesised by two different physiological mechanisms with different response characteristics or control systems. The reduction in egg yolk precursors availability may then result in decreased uptake by the oocyte which in turn results in slower yolk accumulation and oocyte development. If it is, this has a considerable role in the changes of egg production in heat-stressed hens. Furthermore, oestradiol may control not only

hepatic yolk precursor synthesis but also the uptake process at the oocyte (Senior, 1974); as oestradiol and luteinizing hormone are involved in the ovulatory process (Senior and Cunningham, 1974). Indeed the effects of oestradiol administration upon the transport and uptake of vitellogenin and VLDL during heat stress may merit further investigation.

These effects relate to the extent of heat stress induced hyperthermia but are not the main consequence of concomitant decreased food intake. This was examined by studies carried out in **Chapter Five** which evaluated the influence of food intake on laying hens' performance at high (35°C) and thermoneutral (21°C) environmental temperatures. The results of those studies confirmed that decreased egg yolk precursor in the circulating levels were not mainly due to decreased food intake. These studies also provided a better understanding of why the patterns of changes in the egg yolk precursor levels of food restricted hens at thermoneutral condition were not similar to heat-stressed hens. The results also support the proposal that reduced food intake is one response of hen to elevated temperature (Mitchell and MacLeod, 1983) in order to reduce heat production by lowering their metabolic rate. Decreased egg production in heat-stressed birds, however, is clearly not due solely to the reduced food intake as has been suggested. This may involve changes in hormonal balance and function and the regulation of blood flow (Bottje and Harrison, 19984;

The study also confirm that food intake in laying hens is inversely related to ambient temperature as suggested (Sykes, 1979; Fisher and MacLeod, 1985). The results in this study (**Chapter Five**) confirm this finding and even showed that decreased egg production that occurs at high ambient temperature is not entirely due to reduced food intake in heat-stressed hens. These results agree with previous studies (Fuller and Dale, 1979; Fisher and MacLeod, 1985). In addition, reduced food intake is known as one of possible strategies of laying hens in alleviating the effects of heat stress (Mitchell and MacLeod, 1983) besides food withdrawal practice or darkness (Francis *et al.*, 1991). Reduced food intake might also be related to changes in thyroid function (Mitchell and

Raza, 1986; Mitchell and MacLeod, 1988; Denbow, 1995) and the peripheral GH concentration in fasted birds (McMurthy and Johnson, 1988).

Convincing results in pair-fed experiment (**Chapter Five**) showed that birds kept under thermoneutral temperature in this study, which consumed the same amount of food as heat-stressed birds, showed a better laying performance and having higher egg yolk precursor concentrations. Heat-stressed birds reduced their egg production and had lower availability of yolk precursors in the circulation. However, this study does not evaluate the physiological mechanisms how do the factors are inter-correlated. The mechanisms involved are not yet elucidated. This may involve changes in the function and interaction of the thyrotrophic and somatotrophic axis as proposed by Kan (1994). He found an important evidence of possible endocrine basis for the derangement of growth rate induced by chronic heat stress in broiler chickens.

The reduction in precursor availability may then result in decreased uptake by the oocyte, slower yolk accumulation and oocyte development and consequent changes in egg production. Adaptive responses in the oocyte uptake mechanisms may occur concurrently during heat stress but have yet to be characterised

Further studies were carried out as described in **Chapter Six** to determine whether the mechanism mediating reduced egg production in heat-stressed hens involved a change in the rate of uptake of yolk precursors by the oocytes. The new technique in labelling of vitellogenin with ^{65}Zn in fresh laying hen plasma by incubation proved effective. This made possible to monitor ^{65}Zn distribution, elimination, uptake by oocytes and effective recovery by means of γ -counting. As proposed by some other authors changes in blood flow to the reproductive tract appear as soon as the bird suffers from heat stress. Although the number of the birds employed in this study was very small (two hens), the comparison of the results confirmed that much more time is required by a heat-stressed bird to produce an egg. Regardless the number of hens employed and the results of the rate of uptake the study clearly indicated that the novel technique is appropriate for the *in vivo* measurement of vitellogenin uptake by the ovary.

Further studies were undertaken to determine if there are practical methods of improving egg production in heat-stressed birds lead to further studies to find out a possible practical solution (**Chapter Seven**). These studies examined the effects of dietary supplementation with α -tocopherol (vitamin E), a well known biological antioxidant. The findings showed that supplementation of the diet with 500mg/kg vitamin E increases circulating egg yolk precursor concentrations during exposure to heat stress. This condition may be responsible for the apparent improvement in yolk and egg production. There are possibly some oxidative damage in some organs and tissues during the chronic exposure to heat stress. Therefore a supplementation of an antioxidant could play an important role in protecting hepatocytes function during chronic heat stress. It is, therefore, suggested that vitamin E supplementation represents a possible strategy for reducing the effects of heat stress.

If indeed there is a reduction of peripheral blood flow, included to the reproductive organs, in heat-stressed birds as proposed by Akester (1984) and Richards (1976), heat-stressed birds may have less yolk precursors across to their oocyte membranes or in other words they need a longer time to produce an egg which in turn decrease egg production.

There were changes in the triiodothyronine secretions in response to oestrogen stimulation but not in the thyroxin concentration. These observations were not known whether they were linked together or not since thyroxin is the major source of triiodothyronine in the circulation (**Chapter Four**). Administration of oestrogen also induces the circulating levels of egg yolk precursors in both heat-stressed birds and those at thermoneutral temperatures. Heat stress may inhibit yolk precursor synthesis in the liver either by reduction in oestradiol secretion or reduced sensitivity of the hepatocytes to oestrogen stimulation. However, the responses of vitellogenin were not similar with those in VLDL. Even in other section of this study (**Chapters Three and Five**) heat stress alters their concentrations in different patterns. These findings of the study suggest that the two yolk precursors, even synthesised under the same oestrogen action by the

same organ, have different response characteristics or control systems under heat stress conditions. Although the exact mechanisms controlling egg production of heat-stressed hens is still poorly understood, but it is definitely not mainly due to decreased food intake as originally proposed.

In the heat-stressed birds, the reduced plasma egg yolk precursors availability may cause in decreased uptake by the oocyte and/or slower yolk deposition. The ovarian uptake determination was studied in **Chapter Six** shows that the technique is certainly appropriate for the measurement of oocyte vitellogenin uptake *in vivo*. This technique may allow the elucidation of the mechanisms controlling oocyte development and yolk accumulation in response to environmental challenges. The study has achieved the objectives and will form the basis for more thorough validation and improvement of the techniques, which will then constitute an important avenue for progress in the on-going wider programme relating to egg production in tropical climates. These considerations suggest that the technique may not be of particular use in the field, and further validation work would be required to establish its usefulness in any specific circumstance.

It is suggested that not only the environment but also the breeds, in terms of egg yolk precursor synthesis, ovarian uptake and others reproductive hormonal functions, have potential influences in producing an egg. It is not clear that the different responses of commercial line of laying hens to heat stress occurs as the consequence of selection pressure (high laying productivity) or reduced heat tolerance, as both these factors are present in the commercial laying hens.

The study also confirms that vitamin E helps heat-stressed birds to improve their egg production during the exposure to high ambient temperature. The vitamin also increases the availability of egg yolk precursors in the circulation which increases the possibility to be taken up by the oocytes. It is known that vitamin E can affect the laying performance of some hens at high ambient temperatures but the mechanisms are still not

clearly known. Vitamin E may use its reducing agent biochemical properties to prevent oxidation

The comparisons confirmed that much more time is required by a heat-stressed hen to produce an egg. The reduction in mean egg weight per day in the heat-stressed group represents 25-37% decrease compared with control group. Concomitantly a decrease in yolk production was also observed. It thus appears that exposure to heat stress reduces the number of eggs laid, the size or weight of each egg produced and the total yolk production and yolk per egg. The appearance of reduced egg production in this study was associated with reduced availability of egg yolk precursors (vitellogenin and triglyceride rich-VLDL) in the circulation to which it may be attributable. Gilbert *et al.* (1983) suggested that the domestic fowl has two primary control mechanisms regulating ovarian function, i.e. the regulation of the number of follicles initially starting growth and the regulation of the rate of atresia in the small follicles. Under adverse conditions it has another way of modifying the ovulation rate by eliminating the large follicles through atresia (Waddington *et al.* 1985). Such atresia is implied in the decrease of egg production in chronically heat-stressed hens in this study.

Different responses in the egg production of heat stressed birds in some studies reported by other authors may also be affected by the different not only in the dry bulb temperature but also in the relative humidity. Humidity of the ambient air could be a critical factor in heat rejection from the heat stressed birds. This study employed some different dry bulb temperature and humidity combinations (see also **Table 8.1**). This may constitute in the degree of heat loads experiencing by the heat stressed birds. By the use of the hydrometric chart in **Figure 1.8** the heat loads can be worked out based on the humidity/temperature combinations. Different heat load employed to the heat stressed birds constitute in producing different degree of heat stress. One of good indicators of heat stress is body temperature. The different of age of the experimental birds may also be contributable to the different responses to heat stress conditions in particular in the egg yolk precursor performance.

Table 8.1. Different temperature/humidity combinations used in this study .

Temperature °C	Relative humidity (%)	Heat load (mmHg)	Changes in body temperature (°C)	Age of birds (week)
21	50	9.2	-	22
30	80	25.4	1.2	50
32	35	12.3	0.8	35
35	35	14.7	1.06	35
35	35	14.7	0.98	35
35	50	21.3	0.92	44
35	55	23.3	1.20	80
35	60	25.2	1.25	52

8.2. Areas for further research projects

The studies in this thesis have demonstrated that reduction of egg production in chronic heat-stressed hens coincides with a decrease in the egg yolk precursor levels in the circulation. Exogenous oestrogen treatments (**Chapter Four**) shows that heat-stressed birds were capable of producing more eggs concomitant with increased egg yolk precursor in the circulation. This findings suggest that the liver of heat-stressed birds were still capable enough of synthesising egg yolk precursors and hence heat-stressed birds had a better egg production. By having changes in the thyroid hormones in heat-stressed birds, there is a possible assumption that the level of oestrogen in the circulation may be altered during exposure to high ambient temperature. This study, however, does not evaluate the changes of oestrogen in the circulating levels. It is nearly a must that determination of oestrogen concentrations in heat-stressed birds have to determined in the future research in this area. There are some possible mechanisms which may be

involved i.e. (1) oestrogen concentration altered during heat stress (this can be due to decreased its synthesis in the ovary or inhibition by others reproduction hormones), and/or (2) the number of oestrogen receptors in the liver decreased, and/or (3) altered sensitivity of the receptors whilst the numbers are unchanged

This result suggests that future research should, therefore, seek to determine why the oestrogen activities in the liver cells are different between hens under heat-stress and thermoneutral conditions. Better understanding of the reason why the activity in liver cells is different between heat-stressed hens and hens kept under thermoneutral conditions may lead to an assumption that there was a decrease the oestrogen concentration during exposure to high ambient temperature; with or without the decrease other ovarian hormones which may influence the hypothalamus-pituitary axes. Administration of exogenous oestrogen with different dosages may be beneficial to evaluate the degree of reduction in oestrogen in the circulation of heat-stressed hens.

Since the findings in this study also showed that heat stress caused in different degrees of decreased egg production and changes in egg yolk precursor concentrations were not similar either (**Chapters Three, Four and Five**), therefore, it is necessary to determine particular (fixed) heat loads in different ages of hens; and/or to determine the effects of heat stress in particular age of hens with different heat loads.

It is well established that vitamin E (α -tocopherol) is known as an antioxidant. Supplementation vitamin E in the diet in this study shows a considerable effects in improving egg production and increase the plasma concentrations of egg yolk precursor in heat-stressed hens (**Chapter Seven**), although the mechanisms are still not well understood. It, therefore, may be suggested that during exposure to high ambient temperature there is an oxidative damage. This is as proposed by McCay (1985) that there are two of the main methods to prevent oxidative damage; first, radicals are scavenged by vitamin E as a first line of defence; and secondly, glutathione peroxidase destroys any peroxides formed before they can damage the cell. The two defence mechanisms complement each other (Hoekstra, 1975). As α -tocopherol has the highest biological activity (Hennings,

1986; Chen, 1992), therefore incorporation of vitamin E (α -tocopherol) in the diet may help to reduce lipid peroxidation.

Although supplementation of vitamin E results in improved egg production in heat-stressed hens in this study, it is a scientific basic finding. This means that it does require further research in this area to allow the implementation of the finding in the commercial basis. This study applied a concentration of 500 mg/kg diet supplementation. It might not be economically satisfied. The use of the vitamin can be limited by applying the vitamin supplementation in particular period of laying status. Therefore, it is necessary to investigate the role of this vitamin in birds at (1) rearing periods, (2) before and up to the point of lay, and (3) peak of lay. In addition, investigating the effects of this vitamin with different dose of supplementation (from 100 to 750 mg/kg diet) is also suggested.

8.3. Possible benefits of the findings

There are some workers who have raised the hypothesis of a harmful effect of high protein diet under heat stress condition (Waldroup *et al.*, 1976). The explanation here is twofold. One is that excess amino acids in the bloodstream may depressed food intake because of their effect on the hypothalamus. The second reason is the high heat increment of protein: thus a reduction in protein catabolism would result in decrease in heat production and help the bird in maintaining the energy balance in the high temperature condition. Daghir (1995) suggested to have a nutritional manipulation to alleviate the effects of heat stress with a low-protein diet balanced with commercial food-grade methionine and lysine.

There are some technical practices that can be applied in order to improve poultry production in the tropical and subtropical areas. One of them is the use of cooling water to reduce the ambient temperature in poultry houses to prevent irreversible heat prostration occurring. This method will be more efficacious if humidity in the house can also be reduced. A more efficient cooling system to prevent severe heat stress both by

reducing dry bulb temperatures and humidities may be able to achieve better poultry productivity for commercial poultry producers. The use of high nutrient or fat supplementation to compensate for the reduced food intake during exposure to high ambient temperature may cause other problems such as high nutrient food leading to more heat production and high fatty acid diets may cause rancidity problems in food storage especially in the hot and humid tropical areas.

This study offers a practical solution to alleviate the effects of high ambient temperature and/or to improve the egg production in heat-stressed hens. In order to achieve increased laying productivity, commercial poultry producers can use supplemental dietary vitamin E when hens are at high ambient temperatures. Although this requires further studies to determine the most economical approach in terms of the dosage that should be applied and the appropriate time when the vitamin should be supplied, i.e. either when the birds are still growing, before laying or during peak point of lay.

CONCLUSIONS

Arising from the study presented above it can be concluded that:

1. Chronic heat stress in laying hens considerably reduces egg production, yolk size and total daily yolk production.
2. Heat stress reduces the yolk and egg production concomitant with reduced circulation of two major egg yolk precursors i.e. vitellogenin and VLDL in the plasma.

3. With similar degrees of food restriction the thermoneutral birds have better performance in their egg production and total yolk production concomitant with higher plasma concentration of egg yolk precursors compared with heat-stressed birds. The decreased plasma concentrations of vitellogenin and VLDL in heat-stressed birds are not only simply a consequence of reduced food intake.
4. Oestrogen administration *in vivo* significantly ($P<0.01$) increases the availability of vitellogenin in the circulation of both heat-stressed and control (thermoneutral) hens.
5. The results suggest that liver is capable of synthesising both vitellogenin and VLDL but the circulating oestrogen concentration is probably altered during exposure to high ambient temperature.
6. The new technique in labelling of natural vitellogenin present in high concentrations in fresh laying hen plasma by incubation with ^{65}Zn proved significantly effective.
7. The technique is certainly appropriate for the measurement of oocyte vitellogenin uptake *in vivo* and therefore for the elucidation of the mechanisms controlling oocyte development and yolk accumulation in response to environmental challenges.
8. Vitamin E supplementation in the diet represents a possible strategy for reducing the effects of heat stress by improving the egg production performance in heat-stressed hens.

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